University of Alberta

The Toxicity of Condensed Thiophenes and Their Oxidized Derivatives

by

David Todd Seymour ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Microbiology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Toxicity of Condensed Thiophenes and Their Oxidized Derivatives submitted by David Todd Seymour in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Laboratory studies have identified a variety of bacterial metabolites from benzothiophene, methylbenzothiophenes, dibenzothiophene and methydibenzothiophenes. There were three components to this study. One component was measuring the toxicity of twelve organosulfur compounds, many of which are sparingly soluble in water. Saturated solutions of the test compounds were prepared, and suitable dilutions of these were used for the Microtox and *Daphnia magna* assays. However, because few of the solubilities of these compounds are reported in the literature, the second component of this study was to determine the aqueous solubilities of the parent compounds and the synthesized metabolites.

The third component was measuring the changes in toxicity that occurred in batch cultures of *Pseudomonas* strain F grown in the presence of select condensed thiophenes.

The toxicity studies indicated that the oxidized metabolites of the select thiophenes were less toxic than the parent compounds.

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1 Introduction

1.1 Petroleum Contamination and Factors Affecting its Biodegradation

1.1.1 Petroleum Pollution

Increasingly, groundwater, freshwater and marine environments are being contaminated with petroleum due to spills and leakage of underground storage tanks. When most people think of petroleum spills, thoughts of the *Exxon Valdez* come to mind due to extreme media exposure. In actual fact, these spills account for a small amount of petroleum that enters groundwater or makes its way to the shoreline. The National Research Council has estimated that the total annual input of petroleum from all sources into the sea is between 5 and 25 x 10^8 US gallons (79). This works out to be equivalent to 0.09% of total worldwide annual consumption. One hundred million US gallons (or 0.01% of consumption) were related to tanker accidents, which is comparable to natural sources, principally seeps, at 0.7 x 10^8 US gallons (79). When spills occur such as that of the *Exxon Valdez*, it increases public awareness as to the treatment of ecosystems contaminated by petroleum. Increased public pressure accompanied by sensitive analytical instruments has resulted in the necessity of thoroughly cleaned contaminated sites (79). Where once it was thought appropriate to remove the contaminated material to another site, now this practice has fallen out of favor. Researchers are now beginning to look at biodegradation of the contaminant on a large scale.

When oil and refined products are degraded, it is principally done by microorganisms, especially bacteria (79). The general public seem to think that oil and refined products are xenobiotics, chemicals synthesized by humans that have no close natural counterparts, when in actual fact many components of these are very biodegradable. Crude oil is a natural product that is truly organic in all senses of the word (79).

In a National Research Council report, it was estimated that 70×10^6 US gallons of crude oil enter the marine environment every year from natural seeps and by erosion (79). Not all of this oil is seen in the environment, giving indication as to the natural processes that are at work to remove oils from natural seeps.

Soil systems are also very common pollution sites through natural gas and petroleum seeps, accidental seeps and spills, or deliberate disposal of petroleum wastes (13). The clean up of an oil-contaminated soil site is markedly different than that of an aquatic system, but there are certain factors which are common to both.

Petroleum can be divided into four classes (18): the saturates (a general term including *n*- and branched alkanes and cycloalkanes, which excludes the aromatic fraction of oil (48)), the aromatics (hydrocarbons containing one or more benzene rings (48)), the asphaltenes (those components of crude oil that are insoluble in greater than forty volumes of n-pentane (90)) and the resins (the pentane-soluble fraction of a petroleum that, when percolated through fuller's earth or alumina, yields a deep red to brown semisolid material (90)). These different fractions also have differing rates of biodegradation. Perry (78) has ranked hydrocarbons on a basis of decreasing susceptibility to microbial attack: *n* -alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. Zhou and Crawford (96)

have shown that the biodegradation rates are the highest for the saturates, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting very low rates of biodegradation.

1.1.2 Physical State of Oil

When oil is spilled into an aquatic environment it tends to form a slick. Through the action of wind and wave energy, an oil-in-water or water-in-oil, otherwise known as a "mousse", emulsion may form (19). The dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and therefore its availability for microbial attack (64). Eventually the mousse may form large masses, which are otherwise known as plates, resulting in unfavourably low surface-to-volume ratios, which will inhibit biodegradation (22). The formation of tarballs, which are large aggregates of weathered and undegraded oil, restrict access of microorganisms due to their limited surface area.

One of the more important aspects of microbial hydrocarbon uptake is the emulsification of the substrate into small droplets, known as a macroemulsion, or the solubilization of the hydrocarbon in the medium, known as a microemulsion (86). The emulsification or solubilization of a hydrocarbon may occur through agitation, the use of synthetic surfactants or, by the production of extracellular microbial metabolites (biosurfactants) (86).

The movement and distribution of oil and the presence of particulate matter are the key differences between petroleum degradation in soil and aquatic ecosystems (13). The vertical movement of oil into the soil, rather than the horizontal movement accompanying a slick formation, is characteristic of a terrestrial oil spill (64). As oil moves into the soil column, the soil prevents the loss of volatile hydrocarbons, which can be toxic to

microorganisms (64). Particulate matter can reduce, by absorption, the effective toxicity of the components of petroleum, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues (64).

The soil environment is the most complex portion of the biosphere, as indicated by intimately interwoven solid, liquid, and gas phases, a wide range of particle sizes and a tremendous complexity in chemical composition (13). The marine environment, as a whole is fairly uniform, as compared to the composition of the numerous soils found on the planet. The freshwater environment is relatively more variable than the marine environment but still it is not as diversified as a soil system (13).

1.1.3 Temperature

The physical state of petroleum is dependent upon many factors including temperature. The biodegradation of petroleum hydrocarbons is very dependent on its physical state and can occur over a wide range of temperatures (5). Psychrotrophic, mesotrophic, and thermophilic bacteria have been isolated that are able to utilize hydrocarbons (5). The fact that a wide range of temperature-dependent bacteria are able to utilize hydrocarbons is important due to the extensive petroleum contamination, especially in marine ecosystems.

In marine environments the local temperature will have an effect on the rate of petroleum degradation. It has been shown that petroleum degradation was an order of magnitude faster at 25°C than at 5°C (5). Atlas and Bartha (7) have shown that at low temperatures there is an increase in the viscosity of oil, the volatilization of toxic short-chain alkanes is reduced, and their water solubility is increased, delaying the onset on biodegradation.

As seen with other reactions, chemical or biological, the rate of degradation of hydrocarbons decreases as the temperature decreases (64). A possible explanation of this may be attributed to the reduction in the rate of enzymatic activity in most microorganisms at lower temperatures. Accompanying this is an increase in degradation rates up to a maximum point whereby an increase in temperature no longer accompanies an increase in degradation rate (64).

The effects of temperature on the rates of petroleum degradation have to been seen as not being absolute. The effects of temperature differ, depending on the hydrocarbon composition of a petroleum mixture (5). As stated previously, low temperatures slow the rate of volatilization of low-molecular-weight hydrocarbons, some of which are toxic to microorganisms and accordingly the recalcitrance of these toxic components would delay the biodegradation process (5). In a study done by Atlas (4) the biodegradation of seven different crude oils was examined. It was observed that the biodegradation was highly dependent on the composition and on the incubation temperature. The lighter crudes had greater abiotic losses and were more susceptible to biodegradation than heavier oils at 20°C. Interestingly though, at 10°C the light crudes had toxic volatile components which evaporated slowly, inhibiting the microbial degradation of these oils (5). Atlas observed a lag phase before the initiation of hydrocarbon degradation for the lighter oils which was not observed in the heavier oils.

Petroleum contamination in the oceans is quite extensive and of major concern. Due to the low temperature associated with marine environments, the rate of petroleum degradation would not be expected to be at optimal levels. Some microorganisms are capable of growth at near-

zero temperature, but the biochemical activity of these bacteria is much lower than at temperatures near 20-30°C.

1.1.4 Oxygen

The initial process of hydrocarbon biodegradation involves the oxidation of the substrate by oxygenases, thus molecular oxygen is necessary to carry out the metabolism (64). Obviously, the majority of petroleum degradation that occurs in the environment takes place through aerobic processes. Within pristine aquatic systems, both marine and fresh water, there is little likelihood of anoxic conditions (14). The only exception may be during summer or winter stratification in fresh water ponds (14). Within anaerobic basins, the hypolimnion of stratified lakes and benthic sediments, oxygen may severely limit biodegradation (5). As far as sediments are concerned, they are, with the exception of a thin layer at the surface of the sediment, anaerobic (64).

In a process to supply oxygen, Jamison *et al.* (53) used forced aeration of a groundwater supply that had been contaminated with gasoline. It was observed that nutrient supplementation without aeration did not stimulate biodegradation. When both nutrient supplementation and aeration were applied, there was a marked increase in microbial degradation. This method of supplying oxygen is mainly applicable to soil systems. In marine environments the major factors which are responsible for maintaining oxygen levels high enough for biodegradation, are wind and wave action.

It is important to note that if an anaerobic sediment becomes contaminated with petroleum, this does not predetermine that there will be no biodegradation. There will be biodegradation, but it will occur at negligible rates (5). In soil systems, the availability of oxygen is dependent

on the rate of microbial oxygen consumption, the type of soil, whether the soil is waterlogged and the presence of usable substrates which can lead to oxygen depletion (13).

1.1.5 Nutrients

When considering the effects of nutrients on biodegradation rates, most particularly in marine environments, there is debate as to the importance of nutrient availability. When considering nutrients, the most important are fixed nitrogen and phosphorus sources such as NH_4^+ and PO_4^{3-} because in an area of oil contamination, there is an abundance of carbon from the petroleum.

Atlas and Bartha (6) reported that the concentrations of nitrogen and phosphorus in seawater are limiting to microbial hydrocarbon degradation. Conversely, Kinney *et al.* (57) have found that nitrogen and phosphorus are not limiting to hydrocarbon degradation in seawater. According to Atlas (5), the difference seems to be in the perception of biodegradation. Different results would be expected if a study was aimed at the biodegradation of hydrocarbons within an oil slick or the biodegradation of soluble hydrocarbons. It is important to realize that the relative amount of oil contamination will determine whether nitrogen and phosphorus addition is required.

As mentioned previously, there is an overabundance of carbon within an oil slick. But microorganisms also require nitrogen and phosphorus for biomass accumulation (5). In a marine environment, there can be extensive mixing of the oil slick, but this does not mean there will be diffusion of nitrogen and phosphorus into the slick (5). The determining factor seems to be the diffusion rate of nitrogen and phosphorus into the slick. Because

these rates may be exceedingly slow, the ratios of C/N and C/P may be too low to support microbial growth and metabolism (5).

Assuming that nitrogen and phosphorus limit microbial degradation of hydrocarbons, there are methods to improve the rates of degradation. The ratio of C/N and C/P can be adjusted by the addition of nitrogen and phosphorus in the form of fertilizers (64). These include paraffinized urea, octylphosphate, ferric octate, paraffin-supported $MgNH_4PO_4$ and 2-ethylhexyldipolyethylene oxide phosphate (8, 47). One aspect that has to be considered when applying fertilizer to an aquatic system is the dispersion of the fertilizer throughout the water column. This greatly increases the amount of fertilizer required to stimulate biodegradation, especially in areas of high mixing and turbulence such as coastal waters. When water soluble forms of nutrients are added to the open ocean, they can be expected to separate rapidly from the floating oil causing little or no benefit to oil-degrading microorganisms (8). Also, these mineral nutrients may cause algal blooms and cause eutrophication problems (8). Use in the open ocean requires the selection of nitrogen and phosphorus sources that physically adhere to the oil slick and benefit oil-degrading microorganisms selectively (8).

Aquatic systems are not the only areas where nitrogen and phosphorus can become limiting following petroleum contamination. Biodegradation can also be limited in soil systems due to the low amounts of nitrogen and phosphorus (64). Several studies (53, 24, 54) have shown that the addition of urea-phosphate, N-P-K fertilizers and ammonium and phosphate salts have accelerated the biodegradation of crude oil or gasoline in soil and groundwater. Different rate increases, if any at all, can be expected due to the wide variety and composition of different soil systems.

1.1.6 Effect of Prior Exposure

Microbial communities may be exposed to hydrocarbons either through anthropogenic sources such as accidental oil spills, petroleum drilling and transportation, and waste oil disposal, or from natural sources such as seeps (64). The extent of prior exposure can determine how rapidly subsequent hydrocarbon inputs can be degraded. This occurrence, which results from increases in the hydrocarbon-utilizing potential of the community, is known as adaptation (89). Adaptation is defined as a change in the microbial community that increases the rate of transformation of a test compound as a result of a prior exposure to the test compound (89). Spain *et al.* (88) suggest three mechanisms by which adaptation can occur : (i) induction and/or repression of specific enzymes; (ii) genetic changes which result in new metabolic capabilities; and (iii) selective enrichment of microorganisms able to transform the compound or compounds of concern.

It is important to establish the fact that adaptation is not an all-or-none scenario, but rather a qualitative one dependent on preexposure time and concentration (88). Spain *et al* . (88) concluded that during the presence of a contaminant, a particular portion of a population is selected by preexposure to the particular compound.

1.2 Organic Sulfur Compounds in Petroleum and Creosote

1.2.1 Organic Sulfur Compounds

Organic sulfur compounds (OSC), and more specifically sulfur heterocycles, are present in many crude oils and fuels derived from shale oils and coal (35). It has been shown that the OSC are more persistent than the polycyclic aromatic hydrocarbons (11, 12, 40, 75) and with increased use of lower quality, high sulfur content petroleum, their presence in spills

will achieve more recognition. In comparison to the amount of knowledge about the microbial metabolism of hydrocarbons, much less is known about the metabolism of OSC found in petroleum. One of the main reasons for this lack of knowledge of OSC metabolism is due to the lack of commercially available sulfur heterocycles (31). In order to develop knowledge in the areas of chemistry and biological activity, there has to be cooperation between microbiologists and organic chemists. In this situation, organic chemists can synthesize the sulfur heterocycle of interest, and then the microbiologist can perform transformation studies with an organism of choice.

One area of very active research is the microbial process of "biodesulfurization" which has been suggested as a means of selectively removing sulfur compounds from petroleum prior to refining (31). This biodesulfurization process involves the microorganisms of choice oxidizing the OSC yielding water-soluble products which can be separated from the petroleum (31).

1.2.2 Sulfur in Petroleum

The origins of sulfur in petroleum have been attributed to a number of pathways. First and foremost, the biological remains that eventually became petroleum deposits initially contained sulfur. In every living organism, sulfur is vital to life (80). In this manner, those biologicals which gave rise to modern day petroleum deposits, contained sulfur. It is important to note that even though sulfur is essential to life, the quantity in living matter is very small, roughly 1.0 % in a typical *Photobacterium phosphoreum* cell (10).

Commonly there is between 0.1 and 3% of sulfur in some oils (76). The greater portions of sulfur are commonly found in the high boiling

fractions or residues with lesser amounts being present in the aromatic thiophenes such as benzothiophenes or dibenzothiophenes (36). It is unlikely that the small amount of sulfur that is present in living tissue would give rise to such high concentration of sulfur in crude oil. To further strengthen this point, other atoms such as nitrogen and oxygen can be examined. The quantity of these elements in petroleum ranges from essentially none to less than 1% for nitrogen and around 1% for oxygen (80). From these data it is likely that there is another method by which the greater amounts of sulfur are begin deposited into crude oil.

The biogenic reduction of sulfate has been associated with the presence of sulfur above salt domes and currently there are many petroleum scientists who believe that a similar mechanism is responsible for the sulfur in petroleum and natural gases (80).

Depending on the circumstances by which sulfur is incorporated into petroleum, it seems likely that there will be sulfur present in most, if not all crude oils (80). Along this same train of thought, it is reasonable to assume that the amount of sulfur present in crude oils will vary a great deal. Rall *et al.* (80) mention crude oils that have sulfur contents ranging from 0.05 to 13.95 percent.

Thiophene derivatives are especially abundant in crude oils with a high content of aromatics, resins, and asphaltenes. In mature and altered high-sulfur crude oils, dibenzothiophenes are frequently the major thiophenic compound (38). Higher boiling fractions generally contain larger thiophene-containing ring systems (38). Sulfur atoms in asphaltenes, a class of high-molecular-weight components of petroleum, are present as sulfides and in the thiophenes which are not readily accessible to microbial removal

because of the large molecular size, complexity, and colloidal nature of the asphaltenes (38).

1.2.3 Problems Associated With Sulfur Found in Petroleum

As the quantity of high quality, low sulfur crude oils is decreasing, the use of lower quality, high sulfur crude oil is becoming more common. As the use of high sulfur crude increases, the concerns over environmental contamination by these compounds also increases.

Research on the sulfur component of petroleum initially was concerned with the effects of sulfur compounds on the metals used in producing crude oils and on the undesirable characteristics which sulfur compounds impart to the products derived from crude oil (80). The corrosion of metals used in refineries is one of the most costly consequences of sulfur in petroleum. Other undesirable effects of sulfur in petroleum include, but are not limited to, the reduction in octane number of motor fuels, increased engine wear, increased deposits in combustion engines, reduced lubricating-oil life, and atmospheric contamination (80). Acid rain is the result of sulfur dioxide being produced from the combustion of sulfur-containing fuels (35). Catalysts used in oil refineries become useless due the presence of sulfur in crude oils (35). The souring of oil reservoirs through production of hydrogen sulfide costs oil companies several billion dollars every year (16).

One of the more common methods of sulfur removal from crude oil is through chemical methods. This hydrodesulfurization utilizes a catalytic reduction of organic sulfur to hydrogen sulfide (38). A possible alternative to hydrodesulfurization is biodesulfurization.

The microbial metabolism of sulfur compounds is known as "biodesulfurization". Biodesulfurizattion is gaining favour over

hydrodesulfurization because of potential savings from the reduced temperature and pressure requirements and the promise of specific sulfur removal (38). This area of research is concerned with the ability of microorganisms to selectively remove sulfur compounds from petroleum prior to refining (31). The theory behind this is to have the microorganisms of interest oxidize the OSC compounds to water-soluble products which can be separated from the petroleum (31).

Another area of concern is the carcinogenic effects of OSC. Initially, the majority of research was concerned with the effects of polycyclic aromatic hydrocarbons, a known class of environmental carcinogens. The polycyclic aromatic compounds are formed through the inefficient combustion of fossil fuels and other organic matter. The effect of polycyclic aromatic sulfur heterocycles is getting increased attention (26). There have been studies (56, 93) showing the mutagenic and carcinogenic activity of polycyclic aromatic sulfur heterocycles. Research associated with oil spills and chronic pollution have indicated that the polycyclic aromatic sulfur heterocycles may be more resistant to microbial degradation and accumulate to a greater extent than their polycyclic aromatic hydrocarbon analogs (11, 12, 25, 40, 41, 94).

In a study with three- and four-ring polycyclic aromatic sulfur heterocycles, Pelroy *et al.* (77) observed the mutagenicity of some of these compounds. The only three-ring compound that was mutagenic, as determined by the Ames test, was naphtho[1,2-b]thiophene (77). Some of the more mutagenic four-ring compounds included phenanthro[3,4-b]thiophene, anthra[2,1-b]thiophene, anthra[1,2-b]thiophene, anthra[2,3-b]thiophene, phenanthrol[3,2-b]thiophene, and benzo[b]naphtho[2,1-b]thiophene (77).

Another study examined the mutagenicity of methyl analogs of some polycyclic aromatic sulfur heterocycles including dibenzothiophene, benzo[b]naphtho[1,2-*d*]thiophene, benzo[*b*]naphtho[2,1-*d*]thiophene and benzo[*b*]naphtho[2,3-*d*]thiophene (68). McFall *et al.* (68) observed that there was little or no mutagenicity as determined with the *Salmonella*/microsome mutagenicity (Ames) test for many of the polycyclic aromatic sulfur heterocycles. However, when some of the methyl derivatives of these compounds were tested, mutagenicity was observed.

1.2.4 Metabolism of Polycyclic Aromatic Sulfur Heterocycles

It is important to understand the ability of microorganisms to degrade the polycyclic aromatic sulfur heterocycles because it is known that they tend to persist in the environment in areas of acute or chronic pollution (40).

The complete degradation of an organic compound leading to the release of much of the carbon in the form of carbon dioxide, a process known as mineralization, can be examined through two common methods (31). In many metabolism studies, the chosen method is to examine a single microorganism's ability to metabolize a single compound of interest. This is referred to as pure culture-pure substrate studies. This is one of the more effective ways to study microbial degradation of a particular compound because there are few variables to be examined.

Other times a particular organism on its own may not be able to degrade a specific compound. In such circumstances, a consortium of microorganisms may be utilized to degrade this compound. In these mixed culture experiments, the microorganisms must work together to mineralize an organic compound. The basis behind this is that the metabolic end products of one microorganism serve as the substrates for other

microorganisms in the culture (31). Some organic molecules, and commonly polycyclic aromatic sulfur heterocycles, will not serve as a source of energy or carbon for bacteria, but the compounds may be transformed in the presence of other organic compounds which serve as the primary energy source (31). This is known as co-metabolism. In a study with a *Pseudomonas* isolate Foght and Westlake (39) found that this isolate could only degrade dibenzothiophene when an alternate carbon source was provided in the growth medium.

There have been hundreds of organic sulfur compounds identified in crude oil (80), but fewer than twenty were examined in biodegradation studies prior to 1989 (31). The major reason for this lack of investigation is because few of these organic sulfur compounds are commercially available.

Only recently has the metabolism of polycyclic aromatic sulfur heterocycles gained attention. Although the physico-chemical properties of the sulfur analogs are similar to those of the parent polycyclic aromatic hydrocarbons, there metabolism is directed by the sensitivity of the sulfur atom to oxidation (49).

In a eukaryotic model system Jacob *et al.* (50) studied the metabolism of four- and five- ring thiaarenes (benzo[b]naphtho[1,2-d]thiophene, benzo[b]naphtho[2,1-d]thiophene, and benzo[b]naphtho[2,3-d]thiophene as well as triphenyleno[1,12-bcd]thiophene) with liver microsomes of untreated and variously pretreated Wistar rats. The predominant pathway of oxidative metabolism was observed to be through the oxidation of the sulfur atom. The majority of the observed metabolites were sulfones followed by the corresponding sulfoxides. In three benzonaphthothiophenes, ring-oxidation as well as sulfur-oxidation was observed, yielding sulfone phenols.

Takata *et al.* (92) found in a study on the stereochemistry of sulfoxide formation during the enzymatic oxidation of a number of sulfides, that the oxidation to optically active sulfoxides takes place at the less-hindered side of the sulfide.

In a study with *n* -alkyl tetrahydrothiophenes, Fedorak *et al.* (33) found that microbial cultures would either selectively remove the alkyl side chain through a series of beta-oxidations or selectively oxidize the sulfur atom of the compounds to yield the sulfoxide or sulfone. In studies with 2-*n* -dodecyltetrahydrothiophene (DTHT) and 2-*n* -undecyltetrahydrothiophene (UTHT), a number of the major metabolites resulted from the selective removal of the alkyl side chain. Another set of intermediates included the corresponding sulfoxide and sulfones, arising from the selective oxidation of the sulfur atoms of the tetrahydrothiophenes (33). A 28-day incubation experiment resulted in the maximal recovery of tetrahydrothiophene-containing compounds from DTHT or UTHT of approximately 50%. This lack of recovery of the tetrahydrothiophene compounds suggests that the ring structure is susceptible to further microbial attack (33).

Other alkyl-substituted thiophenes were examined for their susceptibility to degradation by pure culture or mixed consortium by Fedorak and Peakman (34). Six alkylthiophenes, 2-hexadecyl-5-methylthiophene, 2-methyl- 2-butyl-5-tridecylthiophene, 2-(3,7-dimethyloctyl)-5-methylthiophene, 2-methyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene and 2-ethyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene were synthesized and used as substrates in biodegradation experiments (34). Generally, the long alkyl side chains were attacked, resulting in the following metabolites accumulating in the medium: 5-methyl-2-thiopheneacetic acid from 2-hexadecyl-5-methylthiophene; 5-methyl-2-thiophenecarboxylic acid from 2-

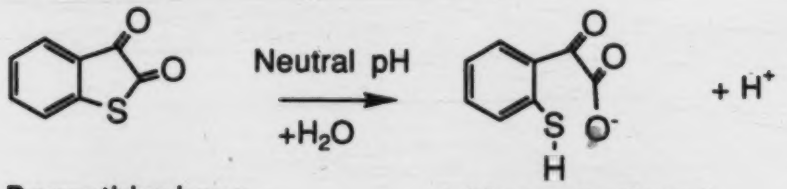
methyl-5-tridecylthiophene; 5-butyl-2-thiophenecarboxylic acid from 2-butyl-5-tridecylthiophene; and 5-ethyl-2-thiopheneacetic acid from 2-ethyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene. The transformations listed here are most likely the result of the degradation of the alkyl side chain through beta-oxidation.

The butyl side chain of 2-butyl-5-tridecylthiophene was not attacked in these studies. Fedorak and Peakman (34) propose that this side chain is too short to be attacked by pure cultures of *n* -alkane-degrading bacteria. There was no detection of ring-oxidation or ring-cleavage products. This does not rule out the possibility of the further degradation of the thiophene ring.

In a study of the metabolism of benzothiophene, Kropp *et al.* (60) found that the transformation products of benzothiophene were often oxidation products of the thiophene ring rather than the cleavage of the aromatic ring. In a cometablism study with benzothiophene or 3-methylbenzothiophene and 1-methylnaphthalene, the major products were benzothiophene-2,3-dione from benzothiophene and the sulfoxide of 3-methylbenzothiophene (32). In order to determine the actual identity of metabolites formed from degradation studies, the observed metabolite was analyzed by gas chromatgraphy-mass spectrometry and then compared to an authentic standard. If the retention time and mass spectrum of the metabolite and the authentic standard are identical in their m/z ratios, then it is possible to identify the metabolite.

Saftic´ *et al.* (82) found that in the cometabolism of methyl-substituted benzothiophenes, the predominant transformation products were sulfoxide and sulfones when the methyl group was on the thiophene ring. Alternately, the major transformation products were 2,3-diones when the methyl group was on the benzene ring (82). Eaton and Nitterauer (27) showed that, at the

neutral pH of the buffer and the toxicity test, the 2,3-diones exist as 2-mercaptophenylglyoxalates as shown below.



Benzothiophene-2,3-dione

2-Mercaptophenyl-glyoxalate

Eaton and Nitterauer (27) observed a wide variety of metabolites from the biotransformation of benzothiophene by an isopropylbenzene-degrading microorganism. These include *trans*-4-[3-hydroxy-2-thienyl]-2-oxobut-3-enoate, *cis*-4,5-dihydroxy-4,5-dihydrobenzothiophene, *trans*-2,3-dihydroxy-2,3-dihydrobenzothiophene, benzothiophene-2,3-dione, 3-hydroxythiophene-2-carboxaldehyde, 2-, 3-, 4-, and 5-hydroxybenzothiophene, 2-mercaptophenylglyoxalate, and 2'-mercaptomandelaldehyde (27).

Four methyldibenzothiophene isomers were synthesized and used in degradation experiments with three *Pseudomonas* isolates that have the ability to degrade dibenzothiophene to 3-hydroxy-2-formylbenzothiophene (HFBT) (83). Saftic´ *et al.* (83) found that a methyl-HFBT and a methylbenzothiophene-2,3-dione were observed from each of the methyldibenzothiophene isomers. This indicates that there is preferential attack on the unsubstituted ring and there was ring cleavage on the ring without the methyl group (83).

Kropp *et al.* (60) observed a variety of metabolites from the bacterial transformation of benzothiophene and six methylbenzothiophene isomers.

When the *Pseudomonas* isolate SB(G) was grown in the presence of benzothiophene, analysis of the culture extract showed the presence of the corresponding sulfoxide and sulfone of benzothiophene as well as a high molecular weight product with a formula that is consistent with $C_{16}H_{10}S$. *Pseudomonas* isolates F and W1 produced the sulfoxide and sulfone of benzothiophene as well as benzothiophene-2,3-dione (60). When the *Pseudomonas* isolate BT1 was grown in the presence of benzothiophene, the only detectable metabolite was benzothiophene-2,3-dione (60).

The growth of these four *Pseudomonas* isolates in the presence of the six isomers of methylbenzothiophene, each in isolation, produced a wide variety of metabolites. These included the corresponding sulfoxide and sulfone, carboxylic acids, methanols, 2,3-diones, *m*-tolyl methyl sulfoxides, *o*-tolyl methyl sulfoxides, and a high molecular weight product with a chemical formula consistent with $C_{18}H_{14}S$, as well as some unidentified products (60).

At this time the amount that is known about the microbial degradation and transformation of polycyclic aromatic sulfur heterocycles is still in its infancy. Even though the amount of knowledge has greatly increased in the last ten years, there is still much to be determined about the degradation of these components of crude oil.

1.3 Toxicity Analysis

One area of interest is the toxicity of these compounds. The majority of toxicity work in the past has focused on the polycyclic aromatic hydrocarbons, and unfortunately there has been little published information on the toxicity of the sulfur analogs of the polycyclic aromatic hydrocarbons. In this project two toxicity methods were utilized to give an indication of the

toxicity of some of the polycyclic aromatic sulfur heterocycles that are commonly found in crude oil. These include the Microtox toxicity testing system and the *Daphnia magna* toxicity test.

1.3.1 Background to Toxicity Analysis

One of the driving forces behind the studies of biotransformations of compounds in petroleum and creosote is to better understand the feasibility of bioremediation in environmental cleanup. Typically, the effectiveness of a bioremediation program is assessed by monitoring the loss of selected compounds, such as the U.S. EPA priority pollutants. However, it is most important to assess the reduction in toxicity of the contaminants, but few studies have addressed this matter.

At a contaminated site, organic contaminants may undergo transformations to other products through biological and chemical reactions (21), but these transformations may not result in detoxification. Using chemical analyses to measure the loss of these parent compounds will not give an indication of detoxification. For example, in one laboratory study, Mueller *et al.* (72) observed the loss of many aromatic compounds from creosote-contaminated soil, but observed little reduction in toxicity. Indeed, some of the intermediate transformation products may be more toxic and/or more mobile than the parent compound (21, 72).

There are numerous methods available to test toxicity (73). Some test organisms are *Daphnia, Ceriodaphnia*, rainbow trout, fathead minnow, and the marine luminescent bacterium *Photobacterium phosphoreum* which is used in the Microtox® system. The results from the Microtox method have been compared to those obtained with other test organisms in many studies (67, 70, 73). In general, the Microtox method is more sensitive than or as

sensitive as the acute lethality tests for pure organic compounds (73). The Microtox method is gaining wide use as a fast, relatively inexpensive procedure for the initial toxicity screening of samples arising from bioremediation processes (2, 21, 23, 67, 72, 91), and of pure compounds (43, 52).

Few toxicity studies have been done with the condensed thiophenes and little is known about the toxicity of the microbial metabolites of these compounds. Among the biochemical transformations of organosulfur compounds that have been reported, the sulfoxides and the sulfones are formed by bacterial cultures (12, 20, 32, 34, 82) and by the cytochrome P450 dependent mixed-function oxidases (48). Some sulfoxides have been shown to be more mutagenic than their parent thiophenes (49). When administered orally to rats and mice, the LC_{50} values of benzothiophene were 1.26 and 0.96 g/kg, respectively (63). The LC_{50} values of benzothiophene sulfone in the same experiments were 3.75 and 1.57 g/kg, respectively, but in contrast to benzothiophene, its sulfone had significant chronic toxicity. In a compilation of toxicity values, the only condensed thiophene commonly found in oil tested was dibenzothiophene (55). Dibenzothiophene had a reported IC_{50} value of 0.122 mg/L (55). For a definition of LC_{50} and IC_{50} see section 1.3.7.

There are many OSC found in crude oil. It is known that the biotransformation of these compounds results in the formation of the respective sulfoxides, sulfones and occasionally, diones (82). If one was assessing the success of bioremediation of a contaminated site by a suitable analytical method (e.g. GC-MS), then the disappearance of chemicals that were initially present would lead to the assumption of a clean site. Whether complete mineralization has occurred is often not examined. The

recalcitrance, mobility, and most importantly, the toxicity of these derivatives has to be examined.

It is not known if the products of the biotransformation of these compounds are more or less toxic than the parent compounds. If the transformation products were more toxic than the parent compounds, then the process of biodegradation of OSC would be more harmful than if the compounds were left alone.

This has led to toxicity testing using the Microtox system. One of the major benefits of this system is the speed at which results are available. This has made the Microtox system very useful for monitoring effluents. Also, the system is simple to use, a small volume of sample is required and it is inexpensive once the system has been purchased. Environment Canada has stated that it is suitable for assessing the short term toxicity of industrial or municipal effluents, leachates, elutrates, mixing zones of surface waters, chemicals, toxicants released from sediments or soils, and material that enters water from a variety of sources (29). The system can also directly test samples of sediment or other semi-solid substances such as municipal or industrial sludge.

An added benefit of this system is the lack of a maintenance culture, such as that needed for the *Daphnia* or rainbow trout tests. This lack of a maintenance culture is because the microorganisms are purchased in the freeze-dried state and can be stored for up to a year before use.

It is extremely important to realize that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection (29). It was proposed by the Inter-Governmental Aquatic Toxicity Group of Canada that there be the development and standardization of a set of single species aquatic toxicity

tests that would be widely acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups.

1.3.2 Detoxification

Detoxification refers to the changes in a molecule of interest that make it less harmful to susceptible species (1). There is a lot of interest in the detoxification of molecules that are harmful or potentially harmful to humans. Microorganisms play a very important role in the detoxification of molecules. The detoxification of molecules by microorganisms can be beneficial to humans, and as bacteria are the lowest trophic level they can prevent some instances of bioaccumulation which could potentially harm humans. Thus in these instances the detoxification plays two important roles. Detoxification is to the benefit of the microorganisms carrying out the transformation if the concentration of the molecule is in the range that suppresses these species (1). This detoxification of molecules plays an important role if the microorganisms use the molecule as a carbon source (1). Microorganisms may also detoxify molecules that are of no apparent benefit to themselves. This is particularly important when these molecules are potentially toxic to higher trophic levels of life, especially to humans.

1.3.3 Aquatic Toxicology

In most biodegradation studies, the emphasis of the fate of compounds eventually involves the aquatic environment. Most of the compounds that are of interest may eventually end up in aquatic systems, including groundwater, freshwater and the marine environment. The biotransformation of compounds to more polar compounds enables these

products to enter aquatic systems as they will be able to move throughout the water table from soil systems. It is in this regard that the focus of toxicity will be aimed at the aquatic environment.

Aquatic systems on the whole are very diverse and a detailed description of these is beyond the scope of this introduction but there are some very important aspects which have to be recognized first. The susceptibility of aquatic ecosystems to chemical contamination depends on such factors as the physical and chemical nature of the compound and its transformation products, the concentration of the chemicals entering the ecosystem, the duration and types of inputs, properties of the ecosystem which would enable it to resist effects of the chemicals, and location of the ecosystem in relation to the release site of the chemicals (81).

A toxicant is an agent that can produce an adverse effect in a biological system, seriously damaging its structure or function or producing death. Toxicants can enter aquatic systems through non-point sources such as agricultural runoff, dredged sediment disposal, and atmospheric fallout. Another source of toxicants include point sources such as discharges from manufacturing plants, hazardous waste disposal sites, and municipal waste water treatment plants (81).

It is important to note that the factor which determines whether a chemical agent is potentially harmful or safe is the relationship between the concentration of the chemical to which an organism is exposed and the duration of the exposure. For any potential toxicant, contact with a biological membrane or system may not produce an adverse effect if the concentration of the chemical is below some minimal effective level. Toxicity is a relative property of a chemical which refers to its potential to have a harmful effect on a living organism (81). For a chemical or its transformation products to have

an adverse effect on an aquatic organism, the compound must come in contact and react with an appropriate site on the organism at a high enough concentration and for a sufficient amount of time (81). Naturally the concentration and time will vary from chemical to chemical, the species being examined, and the severity of the effect. This contact-reaction between the organism and the chemical is called exposure (81).

In an aquatic system, those chemicals which are more water soluble are more readily available to aquatic organisms than chemicals which are highly water insoluble. The water insoluble chemical would likely be bound to particulate matter or found in the sediment. This is not to say that these water insoluble chemicals cannot produce a toxic response. They may be ingested along with particulate matter by an aquatic organism and then cause a toxic response. Those chemicals that are dissolved in the aquatic system can enter organisms through the general body surface, gills or mouth.

Along with the wide effects of chemicals, there are variations of response to the effects of chemicals between and within species. Rates and pattern of metabolism and excretion between different species can affect susceptibility. Also there are genetic variations within a given species that will result in variations of the effect of a certain chemical (81). It is known that young organisms are more susceptible to the effects of a chemical than an adult. This is an important factor in the *Daphnia* toxicity test as the test organisms are neonates which are less than 24 h old. Dietary factors which result in a change in body composition, physiological and biochemical functions will affect the way an organism responds to a particular chemical (81). Another important factor is the relative health of the organism. If the

organism of interest is stressed then it would be more susceptible to a toxicant.

The composition of a particular wastewater can also affect its toxicity to a particular organism. There are instances where the makeup of a particular wastewater contains a large variety of compounds. These compounds may increase or decrease the toxicity of this wastewater and thus give an inaccurate assessment of the wastewater when the sample is analyzed in fractions based on polarity or water solubility for example. There may be carry-over from one fraction to another, and thereby altered toxicity.

One of the more important aspects to consider is the testing of chemicals in isolation. This is the chosen method for many toxicologists because the number of variables are reduced. But it is important to be aware that these chemicals may act in a different fashion when they are in the environment or are associated with similar compounds for example, arising from a similar manufacturing process. The solubility, vapor pressure and pH of the chemical will also affect the toxic response of the chemical to a particular organism.

There are instances whereby the biotransformation of a chemical may yield a product that is more toxic than the parent compound. Mercury is the most widely known compound that is more toxic after anaerobic microbial transformation to the more toxic compounds methyl and dimethyl mercury (81). This then makes the mercury more soluble and it is mobilized from the sediment becoming mixed in the water column. The more soluble forms accumulate in bacteria, which are in turn consumed by higher organisms thus leading to an increase in concentration up through the food chain (81). This process is known as bioaccumulation.

The organophosphorus insecticides containing a P=S double bond are commonly broken down in soil systems to products that contain a P=O double bond (42). Gälli *et al.* (42) found that the P=O analog of disulfoton was 6-times and the P=O analog of thiometon was 75-times more toxic than their respective parent compounds (42).

In a study with a variety of bacterial isolates that were able to degrade dibenzothiophene, Monticello *et al.* (71) found that the products of dibenzothiophene degradation inhibited growth of the isolates, as well as their oxidation of dibenzothiophene. Mueller *et al.* (72) observed little decrease in toxicity and teratogenicity of a biotreated groundwater that was contaminated with creosote and pentachlorophenol. These findings suggest that in some instances the transformation products are more toxic than the parent compound. These cases of increased toxicity have to be considered when a bioremediation process is undertaken. If it is possible that the transformation products are more toxic and potentially more harmful to the environment, then the question becomes whether to stimulate the natural bioremediation process. If there is the chance for toxic byproducts to be formed, then it may be best to leave the native population to remediate the spill at natural rates.

1.3.4 General Mechanisms of Toxicity

All chemicals produce their toxic effects via alterations in normal cellular biochemistry and physiology. There are many ways in which chemicals can interfere with normal biochemistry and physiology. One such mode of action is related to receptor-ligand interactions and stereoselective actions of chemicals. Receptors are macromolecular components of tissues with which a drug or chemical (ligand) interacts to produce its characteristic

biological effects. The ligand may be an endogenous substance that interacts with the receptor to produce a normal physiological response, or it may be an exogenous substance that may either activate or block the response. Receptor-ligand interactions are generally highly stereospecific, and small changes in chemical structure can drastically reduce or eliminate the effect. The adverse effects of many chemicals are related directly to their ability to interfere with normal receptor-ligand interactions.

Many chemicals produce their adverse effects by interfering with the oxidation of carbohydrates to produce adenosine triphosphate. This can be achieved through the blocking of effective delivery of oxygen to tissues. In mammals, the chemical oxidation of the iron in hemoglobin (methemoglobin) by nitrites also interferes with oxygen delivery, as methemoglobin does not effectively bind oxygen.

Many toxic agents produce their effect by binding to the active sites of enzymes or proteins that are critical to cellular function. For example, hydrogen cyanide binds well to the ferric iron atom in cytochrome a + a_3 , which blocks the terminal event in electron transport.

There are many more modes of action beyond the scope of this review which specifically are focused on organ systems of higher organisms. It is important to acknowledge the fact that as some chemicals can be characterized by actions on specific areas, there may also be multiple sites and mechanisms of actions.

1.3.5 Toxicity Testing with *Daphnia magna*

The *Daphnia* test uses the organism known as the "waterflea", a small freshwater crustacean of the Order Cladocera. This organism is found in ponds and lakes of North America, and plays an important part in aquatic

communities (30). Some of their advantages, in addition to toxicity testing at a higher trophic level than the Microtox test, include small size, short life cycles, sensitivity to a broad range of aquatic contaminants, and ease of culturing in laboratories (30). The *Daphnia* toxicity test is an approved test for toxicity analysis in numerous countries, and because of this it is one of the more widely performed aquatic toxicity tests throughout the world. In general, the species used are those that are freshwater-acclimated. There are instances where the organisms are acclimated to seawater for studies of samples from marine or estuarine environments (85).

Generally the two species that are used for this test are *Daphnia magna* and *Daphnia pulex*. The choice of species depends on the hardness of the dilution water. *D. magna* can be used if the hardness is greater than or equal to 80 mg/L $CaCO_3$. When the hardness of the water is below 80 mg/L then *D. pulex* is the organism of choice (85). When performing a toxicity test with daphnids, only neonates that are less than 24 h old can be used as the test organisms.

For the culturing of daphnids there are strict guidelines imposed by Environment Canada. Water has to be uncontaminated groundwater, dechlorinated municipal drinking water or reconstituted water adjusted to the desired hardness (30). It is important that there is daily monitoring of temperature (20±2°C), dissolved oxygen (> 60% air saturation), pH (6.5 to 8.5), and hardness (80 to 250 mg/L of $CaCO_3$) of the water used for culturing the organisms (85). A cool white fluorescent light is suitable for lighting as long as the photoperiod is 16±1h: 8±1 h, light : dark cycle (30).

One of the most important aspects of toxicity testing is the requirement for thoroughly cleaned and rinsed test vessels, measurement and stirring devices. Reconstituted water can be used for dilution water as long as it is

within the limits of hardness outlined by Environment Canada (85). Any number of test solutions and concentrations can be used depending on the test. It should be noted that for some specific tests there are strict guidelines as to the number and concentrations of test solutions. An appropriate geometric dilution series, in which each successive concentration is about 50% of the previous one (100, 50, 25, 12.5, 6.3%), is commonly used to aid in the precise calculation of the LC_{50} (85). Replicates can be employed if desired.

Adjustment of sample pH can be made with hydrochloric acid or sodium hydroxide if necessary. This can result in alteration of the sample and should be used only when necessary. The temperature of the dilution water and sample should be adjusted to the test temperature (20±2°C) prior to use (85).

There are many methods designed by Environment Canada for the isolation of the neonates (85), but the daily separation of neonates from the *Daphnia* culture allows for daily sampling and excludes the requirement of separating the adults from the maintenance culture prior to testing in order to obtain neonates that are less than 24-h-old. Temperature, pH, salinity and dissolved oxygen should be checked in each concentration before starting the test (85).

For each concentration in the test, an equal number of neonates are added to the flask. Usually no less than ten are added to each flask but it is equally important that the loading density of no more than one neonate per 15 mL of reconstituted water, is not exceeded (85). Neonates should be transferred to the test flasks as quickly as possible, with as little carry over water as possible and without undue stress on the organisms (85).

1.3.6 Reported Literature Values of Polycyclic Aromatic Sulfur Heterocycles with *Daphnia magna*

Eastmond *et al.* (26) compared a series of polycyclic aromatic sulfur heterocycles to sterically and structurally similar polycyclic aromatic hydrocarbons for toxicity, bioconcentration, and elimination in *D. magna*. In this study, a LC_{50} value of 318 mg/L was reported for thiophene, 63.7 mg/L for benzothiophene, 0.466 mg/L for dibenzothiophene and 0.22 mg/L for benzo[*b*]naphtho[1,2-*d*]thiophene.

A statistically significant difference was observed between the accumulation curves of benzothiophene and naphthalene in *D. magna*. Elimination curves revealed that naphthalene was eliminated much more rapidly than benzothiophene (26). In contrast to benzothiophene and naphthalene, there was no significant difference in the accumulation curves of dibenzothiophene and phenanthrene (26). There was a significant difference in the elimination curves of dibenzothiophene and phenanthrene, with phenanthrene being eliminated more slowly than dibenzothiophene (26). Eastmond *et al.* (26) concluded that the polycyclic aromatic sulfur heterocycles generally bioaccumulate to a greater extent than their sterically and structurally similar polycyclic aromatic hydrocarbon analogs.

1.3.7 Toxicity Testing with Microtox

The marine bacterium *Photobacterium phosphoreum* NRRL B-11177 is used in this test to determine the toxicity of samples. This organism emits light as the result of normal metabolic processes, and the light is then measured with a standard photodetection device under specified conditions (29). Species of the *Photobacterium* genus are Gram negative, facultatively aerobic, plump, straight rods (10). They are motile by one to three

unsheathed polar flagella, yet there are some that are nonmotile. They are common to the marine environment. Two species, *P. phosphoreum* and *P. leiognathi* are able to emit light of a blue-green color, a property not found in strains of *P. angustrum*. The reaction sequence is similar in all prokaryotes and involves the enzyme luciferase (10). The reaction catalyzed by luciferase involves the luminescent oxidation by molecular oxygen of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde (10). The reaction can be expressed as

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} h\nu + FMN + RCOOH$$

and constitutes a bypass of the electron transport chain since the electrons are shunted from flavin to molecular oxygen without involvement of cytochromes (10).

As mentioned previously, the Microtox system measures the light output of the luminescent bacteria after they have been challenged by a sample, and compares it to the light output of a control that contains no sample (69). A difference in light output is attributed to the effect of the sample on the organisms (69). The extent of light loss, which is indicative of metabolic inhibition in the test organisms, indicates the degree of toxicity of the sample (69).

Gamma (Γ) is the ratio of light lost by the test organisms after exposure to a sample to the light remaining after that exposure (69). Gamma is the measure of light loss used in calculating the IC_{50} and is calculated individually for each cuvette which contains some of the sample being tested. The most common inhibiting concentration reported is the IC_{50}, the concentration at which Gamma equals 1.0, that is, the light lost equals the

light remaining. When calculating Gamma it is first necessary to calculate the Correction Factor. The Correction Factor (R_t) is the fraction obtained when the light output of the Control (Blank) remaining after time t is divided by the initial light output of the untreated Control (Blank) to account for natural decline in light production during the time period of the test:

$$R_t = I_{t,blank} / I_{o,blank}$$

The Gamma value (Γ) is the ratio of the light lost at time t to the light remaining at time t for a given sample concentration, corrected for the change in light output of the control, R_t:

$$\Gamma_t = R_t(I_{o,sample} - I_{t,sample})/ I_{t,sample}$$

The effect measured by the Microtox system, light loss, is a rate of biological activity, rather than a count of organisms affected, which is referred to as quantal data (69). The light produced by the test organism is a byproduct of respiration, and thus reflects changes in respiration. The light measurement integrates the response of approximately one million individual organisms to a toxicant or a control. This bacterium is referred to as "Microtox Reagent" (69), which is composed of the bioluminescent bacteria that were grown under optimal conditions, harvested, and then lyophilized. Before a series of tests, the Reagent is rehydrated with Microtox Reconstitution Solution to provide a ready-to-use cell suspension (69).

The Microtox system has been commercially available since 1978 and now is used worldwide. Some of the benefits of this system are ease of use, short time required to determine results, and relatively inexpensive operating costs. Once the system has been purchased, the costs of individual tests are less than other widely used biological tests such as the *Daphnia* or rainbow trout tests. This feature alone makes the Microtox system very appealing to agencies where there is limited space available for

maintenance cultures. The lack of a maintenance culture also makes it possible for mobile Microtox testing labs to be established.

The test organism *P. phosphoreum* is a marine microorganism, and the Microtox test is normally carried out at greater than 2% salinity by adding a salinity-adjusting solution to the sample and using a dilution water that has a salinity of 2% (29). The amount of light produced in seawater is similar to the amount produced in the test conditions. It is important that the test be carried out at a salinity greater than 2% (29). Solid NaCl may also be used to adjust the salinity of the sample under certain conditions (69).

In a thorough study, Munkittrick *et al.* (73) have shown that the Microtox test provides similar results to those of freshwater organisms. Thus results from the Microtox system can be assumed to be reliable for both freshwater and marine systems. If a sample has to be tested in fresh water there are methods to accommodate the osmotic requirements of the Reagent (29).

As with the wide variety of samples, the effects of the samples may also vary quite considerably. Some compounds may produce immediate effects on the test organisms whereas other compounds may require a longer period of time to produce toxic effects. Accordingly, light levels are commonly read at specific intervals. The most common readings are taken at 5 and 15 min (69), but the test is not limited to these time intervals. Readings may be taken at 30 min, for example.

The effective concentration of a sample at which the light output of the Reagent is reduced by a specified percentage is the endpoint for the Microtox test. The EC_{50} (t, T) is the effective concentration of a sample causing a 50% reduction in the reagent light output under defined conditions of exposure time (t) and test temperature (T) (69). Although EC_{50} is the most

common value presented in reports, other effective concentrations may be generated from the Microtox test. According to Environment Canada (29), LC_{50} (the median lethal concentration) is the concentration of a material in water that is estimated to be lethal to 50% of test organisms after a defined period of exposure. EC_{50} is the concentration of a substance in water that is estimated to cause a specified effect in 50% of the individuals exposed to that concentration (29).In a similar manner as LC_{50}, EC_{50} refers to a quantal effect since each exposed individual must be classified as either showing the effect or not showing it (29). Environment Canada states that the effect must be specified, and often also the exposure time. It is important to recognize that this term does not apply to a percent reduction in some rate or process in an organism or a group of organisms (29). Under Environment Canada protocols, it is recommended that this terminology not be used in the Microtox test; instead an inhibiting concentration (IC) value should be used to describe Microtox results.

An IC*p* value is the inhibiting concentration for a (specified) percent (*p*) effect (29). This value gives a point estimate of the concentration of a specific sample that would result in a designated percent (50%) impairment in a quantitative biological function such as light production by bacteria relative to a control (29). If a change in rate is being examined in a test, such as reproduction, growth or respiration, Environment Canada recommends the use of IC values (29).

As with all toxicity tests there are some limitations. With the Microtox test, all manipulations of the sample and the bacteria are manual. There is variability expected from the operator in reconstituting the bacteria, and in handling the micropipettor and solutions (29). The samples themselves may be the cause of variation. If the sample has a lot of color, especially red or

brown, there may be interference with light transmission which will then skew the toxicity interpretations (29). Microtox recommends a color correction procedure to remedy this problem (69). This is particularly important with pulp mill effluents, which tend to be quite brown in color. Samples that are very turbid may also affect light transmission and thus increase the toxicity estimate (29). Environment Canada suggests that the color correction procedure may correct this problem of turbidity in some instances, while in other cases centrifugation of the sample may be the more appropriate method (29).

The Microtox tests are performed with either a Model 2055 or Model 500 photometric analyzer (29). The analyzer is more than just a device for measuring light output from test cuvettes, it is designed to hold all the test cuvettes and maintain them at a constant temperature of 15±0.3°C. There is one well specifically designed to hold the reconstituted bacteria at a constant temperature of 5°C (29).

The Microtox test system recently has received increased attention. The ease of use and relative inexpense of this system make it very attractive to testing centers and regulatory agencies. The system has been used increasingly for testing pulp mill effluents (37, 67), wastewaters (3), sediment analyses (23, 45, 46, 51), biodegradation studies (70, 72, 91), and a variety of chemicals (9, 17, 28, 87).

1.3.8 Comparison of Microtox to other Toxicity Tests.

Firth and Backman (37) found good correlation between Microtox and *Cerodaphnia* toxicity tests when examining pulp mill wastewaters. In this study the Microtox test proved to be a useful screening test for other toxicity tests.

In a study examining the toxicity of biodegraded pentachlorophenol, Middaugh *et al.* (70) observed a non-toxic response with the Microtox method, but the same samples proved to be embryotoxic or teratogenic to embryonic inland silversides, *Menidia beryllina*.

Phenol is one of the reference toxicants for the Microtox system. Microbics (69) recommends that an IC_{50} value of 13 to 26 mg/L should be expected at 15°C. In a review of the health risks associated with phenol, Babich and Davis (9) found that the LC_{50} values were quite varied among different organisms when exposed to phenol. Of particular interest is *D. magna*. In a 48-h static test, the LC_{50} was 9.6 mg/L (9).

In a study examining the toxicity of some organophosphate insecticides, including thiometon, fenitrothion and disulfoton, Gälli *et al.* (42) found a difference between *D. magna* and *P. photobacterium*. In that study it was found that *D. magna* was, with the exception of thiometon, 200 to 750,000-times more sensitive to the three insecticides tested than the Microtox test (42).

One of the more extensive comparisons of Microtox to other toxicity test was carried out by Munkittrick *et al.* (73). From that study, there were some notable conclusions drawn. The Microtox test was as sensitive as acute lethality tests to most pure organics and was more sensitive to complex compounds such as multichlorinated benzenes, phenols, and ethanols, while being less sensitive to cyanide, chloroform, and phenol (73). The Microtox method was less sensitive than rainbow trout and fathead minnow test and not as sensitive as *Daphnia* to inorganics (73). When insecticides, herbicides, pharmaceutical and textile effluents were examined, the Microtox system was less sensitive than the acute lethality tests (73). One of the major conclusions from that report was the

recommendation that the Microtox system be used as a screening test to examine the toxicity of environmental samples. Then a more thorough analysis can be followed as necessary (73).

1.4 Objective

The objective of this study was to compare the toxicities of selected benzothiophenes and dibenzothiophene with the toxicities of some microbial metabolites of these compounds.

The initial part of this project focused on the toxicity determination of individual sulfur compounds using the Microtox bioassay. Preliminary assays used the compounds dissolved in methanol or dimethylsulfoxide to enhance their solubilities in the Microtox test solution. After it was determined that the IC_{50} values of many of the OSC were below their water solubility, the toxicities of these compounds were determined in aqueous solutions, without an organic solvent.

To improve the reproducibility of the concentrations of the OSC used in the Microtox assays, saturated aqueous solutions of these compounds were prepared and diluted for the bioassays. The aqueous solubilities of the individual OSC were determined by a high performance liquid chromatography (HPLC) method. Once the solubility was determined, it was possible to calculate concentrations of the diluted solutions to be used in the toxicity tests. The *D. magna* toxicity test was employed to determine the toxicity of the individual sulfur compounds to an organism of a higher trophic level.

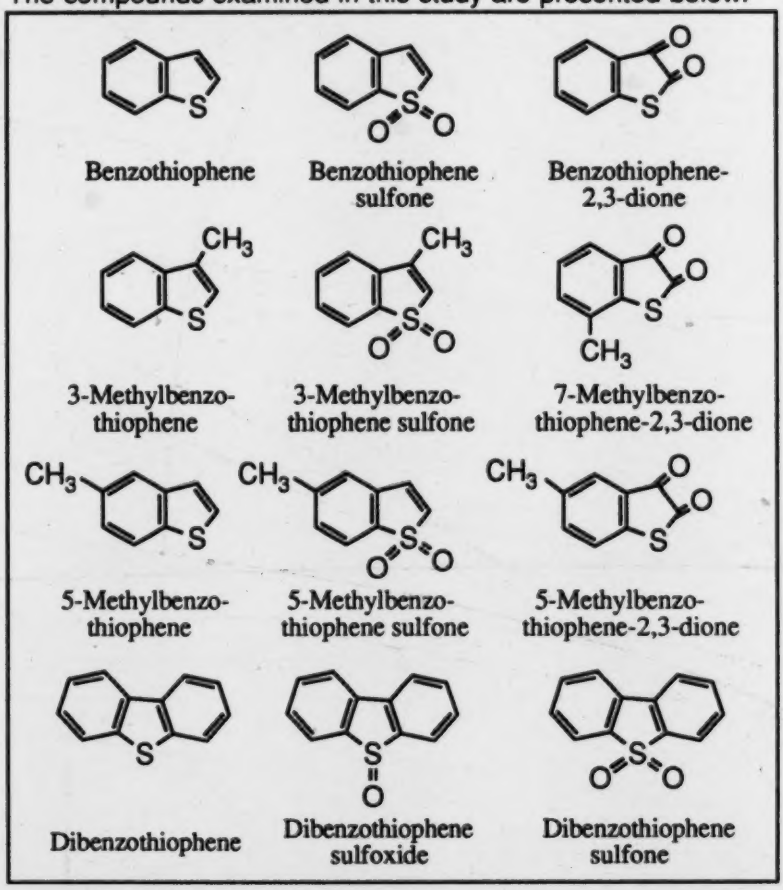
The final component of the study was measuring the changes in toxicity that occurred in batch cultures of *Pseudomonas* strain F grown in the presence of select condensed thiophenes. This was done because some of

the metabolites identified in previous biodegradation studies were not available for toxicity testing. Thus, active bacterial cultures were used to produce these metabolites, and changes in the toxicities of the culture medium were then measured.

2 Methods and Materials

2.1 Organosulfur Compounds

The compounds examined in this study are presented below.



The following compounds were commercially available: 3-methylbenzothiophene and 5-methylbenzothiophene, from Lancaster Synthesis (Windham, NH); benzothiophene and dibenzothiophene sulfone from Fluka (Buchs, Switzerland). Sulfones of 3-methylbenzothiophene and 5-methylbenzothiophene were prepared by boiling the parent compounds with H_2O_2 in acetic acid for 2 h.

Early studies used dibenzothiophene sulfoxide from Fluka. However, cool on-column gas chromatography-mass spectrometric (GC-MS) analysis

of the commercially available preparation showed that it contained about 50% sulfoxide and 50% sulfone. Cool on-column gas chromatography-mass spectrometric (GC-MS) analysis was performed by Sandra Kenefick. Thus, the sulfoxide was synthesized by the method of Castrillón (15) by Dave Bressler, and GC-MS analysis showed this preparation to be approximately 99% pure. The newly synthesized materials were used in toxicity tests in the latter part of the project. 2,3-Diones of benzothiophene, 5-methylbenzothiophene and 7-methylbenzothiophene were synthesized by the method of Hannoun *et al.* (44).

2.2 Determining Aqueous Solubilities of Organosulfur Compounds by High Performance Liquid Chromatography

2.2.1 Preparation of Saturated Aqueous Solutions

Saturated solutions of each test compound were prepared by adding approximately 200 mL of Milli-Q water to a screw-capped 500-mL Erlenmeyer flask. Distilled water passed through a Millipore (Millipore Corp., Bedford, MI) "Milli-Q Water Purification System" is referred to as Milli-Q water. Then a sufficient amount of each compound was added to individual flasks so that no more of the compound was seen to dissolve. To ensure a saturated solution, the flasks were placed on a rotary shaker (Labline Instruments Inc., Melrose Park, IL) overnight at 250 rpm at 28°C. If all of the compound had dissolved, more was added to the solution and the flask was placed on the shaker again overnight. This procedure was continued until the solubility of the compound was exceeded. The solutions were equilibrated to room temperature (24±2°C) prior to HPLC analysis.

2.2.2 HPLC Analysis of Organosulfur and Aromatic Compounds

For toxicity analysis, dilutions of saturated solutions were used. Thus it was important to know the aqueous solubilities of the compounds being tested. Aqueous solutions were prepared and absorbance maxima were measured and used for HPLC analysis.

For analysis of aqueous solubilities, a Hewlett Packard 1050 HPLC with a Hewlett Packard LiChromspher 100 RP-18 column (5 μm, 125 mm x 4 mm) was used. The mobile phase was acetonitrile:Milli-Q water (75:25) and the flow rate was 1.0 mL/min The eluting peaks from the HPLC were detected at the following wavelengths: 240 nm for biphenyl, phenanthrene, dibenzothiophene sulfoxide, dibenzothiophene sulfone, benzothiophene sulfone, and 3-methylbenzothiophene sulfone; 255 nm for dibenzothiophene; and 260 nm for benzothiophene and 3-methylbenzothiophene. To prepare calibration curves, five to seven solutions at different concentrations were prepared for each test compound in acetonitrile. Fifty-microlitre samples of these were analyzed in triplicate by HPLC.

From the standard curves of each of the compounds tested, the aqueous solubilities were determined. Initially the first chemicals tested were ones that had published values for their aqueous solubilities.

2.2.3 Preparation of saturated solutions for solubility determination.

The preparation of the saturated solutions required adding an excess amount of chemical to a flask containing water. Some of these preparations resulted in the formation of suspensions as there tended to be a lot of suspended particles in some of the prepared solutions. In order to reduce

the likelihood of drawing up suspended particles along with the aqueous solution, three samples were analyzed of each saturated solution.

One sample was centrifuged in glass test tubes (13 mm x 100 mm). A 2-mL sample was added to the test tubes and centrifuged at 7000 rpm in a Model CL International Clinical Centrifuge (International Equipment Co., Needham Heights, MA) for 10 min at room temperature. Then three 50-µL samples from the supernatant were analyzed by HPLC. Another sample from the individual solutions was centrifuged in a plastic microfuge tube. One millilitre of the sample was added to a 1.5-mL microfuge tube (Rose Scientific Ltd., Edmonton, AB) and centrifuged in a Denver Instrument Force 7 (Denver Instrument Co., Arvada, CO) microcentrifuge at 10,000 rpm at room temperature. Three 50-µL samples from the supernatant were analyzed by HPLC. The third sample was taken directly from the original suspension that had been allowed to settle for over an hour, and triplicate 50-µL samples were analyzed by HPLC.

Two types of centrifuge tubes were used in this analysis, glass and plastic. The two types were used because it was suspected that some of these thiophenes would adhere to the walls of the plastic microfuge tube. The use of glass tubes would negate this problem in that it is unlikely that these compounds would adhere to the glass.

2.3 Microtox Analysis

For toxicity testing with the Microtox system, a model 500 analyzer (Microbics, Carlsbad, CA) was used. All supplies and materials were purchased from Microbics.

2.3.1 Test Solutions used for Microtox Assays

For the first part of the project, some of the commercially available OSC found in petroleum and some of their microbial metabolites were tested for their toxicity. Initially, the chemicals were dissolved in methanol (British Drug House Inc., Toronto, ON). The reasoning behind this choice of solvent is that, unlike dimethyl sulfoxide (DMSO), it does not contain sulfur. During the early stages of this project, it was planned that extracts of cultures grown in the presence of condensed thiophenes would be tested in a methanol solution, and a portion of this solution would be analyzed for sulfur content, which would be used as the basis for comparing results from different experiments. This would be impossible with DMSO solutions because the sulfur present in the solvent would negate the use of sulfur analysis as a measure of the amount of sulfur originating from the condensed thiophene.

Due to poor solubility of the compounds in methanol, solutions could not be made that would give accurate toxicity values. Solutions prepared in DMSO (British Drug House Inc.,) were then examined for toxicity. Two parallel sets of solutions were prepared for Microtox analysis. An initial solution was prepared and this was tested twice to assess reproducibility. Then another solution was prepared that was of a similar concentration and tested as another check of reproducibility of the method.

At neutral pH, the 2,3-diones were sufficiently soluble that solutions of known concentration could be prepared. To ensure adequate buffering near pH 7, the B+N8P medium described by Kropp *et al.* (61) was used to dissolve the 2,3-diones. This medium contained (per 900 mL) NH_4Cl, 1.0 g; Na_2SO_4, 2.0 g; KNO_3, 2.0 g; $FeSO_4 \cdot 7H_2O$, trace; trace metal solution (32), 1 mL. To this was added 100 mL of a buffer prepared by adding a solution of

KH_2PO_4 (4 g/100 mL) into a solution of K_2HPO_4 (4 g/100 mL) until the medium pH was 7.0.

2.3.2 Basic Microtox Test Protocol

Before the start of any tests, a phenol standard was tested in order to confirm the performance of the complete Microtox method (69). Phenol ordinarily exhibits an IC_{50} between 13 and 26 mg/L (69), and a solution of 100 mg/L was used to prepare dilutions for these tests.

Prior to testing, a vial of Microtox Reagent was reconstituted by placing a clean, unused cuvette (Microbics) in the reagent well. The Reagent well maintains a temperature of 5°C (69). To this cuvette 1000 µL of Microtox Reconstitution Solution (Microbics) was added and allowed to cool to 5°C. While the Reconstitution Solution was cooling, the remainder of the test was prepared.

For one Basic test, a total of 10 clean, unused cuvettes were used, plus the one used for the reconstituted bacteria. The Model 500 Microtox testing device has the capacity of running three Basic tests simultaneously. There are 30 wells arranged in six rows of five wells that are kept at a temperature of 15 ±1°C. The rows are labelled A through F, and across each row the wells are labelled 1 through 5. Thus well A1 is in the uppermost lefthand corner, and well F5 is in the lowermost righthand corner.

A total of ten cuvettes were used for each Basic test, placed in the first two rows of the wells (A1 - A5, B1-B5). One thousand microliters of Microtox diluent was added to each of the cuvettes in wells B1 - B5 (67). Then 1000 µL of Microtox Diluent was added to each cuvette in wells A1 through A4 (69). Two hundred fifty microliters of Microtox Osmotic Adjustment Solution (MOAS) was pipetted into the cuvette in well A5 (69). After addition of

MOAS to A5, 2500 µL of the test sample was added to the cuvette in well A5 and the sample was mixed by filling and dispensing the pipettor 3-4 times (69).

A series of two-fold dilutions was then performed by first transferring 1000 µL from A5 to A4 and the resulting contents of the cuvette in well A4 were mixed as described above (69). One thousand microliters was transferred from A4 to A3, mixed, and finally 1000 µL was transferred from A3 to A2 and mixed. To maintain a constant volume throughout all the cuvettes in row A, 1000 µL was discarded from cuvette A2 and 750 µL was discarded from cuvette A5. It was then necessary to wait for 5 min in order for the temperature to equilibrate in the cuvettes in row A (69).

The cooled reconstitution solution was poured as quickly as possible into the Reagent (bacteria) vial. Then the Reagent was swirled 3-4 times and poured back into the cuvette, and the cuvette was placed back in the reagent well (69). The reconstituted Reagent was mixed 20 times using a 500 µL pipettor. After mixing, 10 µL of the reconstituted reagent was added with a 10 µL pipettor to each cuvette in wells B1 through B5 and was mixed by shaking each cuvette 2-3 times. It was then necessary to wait for 15 min for Reagent stabilization (69).

After the 15-min stabilization period, the light readings were taken for each of the solutions in row B. The supplied software (Microbics) records the initial light output for individual cuvettes. Immediately after the initial light readings were taken, another series of transfers were performed. The following 500 µL transfers were made: A1 to B1, A2 to B2, A3 to B3, A4 to B4, and A5 to B5. This gave final solution concentrations of 45%, 22.5%, 11.25%, 5.625%, and 2.8%. After each transfer, the sample was mixed 2-3 times with the 500-µL pipettor.

At predetermined intervals (e.g. 5 and 15 min) light readings were taken of the solutions in cuvettes B1 through B5. Once all the light reading had been recorded, the computer software generates an EC_{50} value, referred to as IC_{50} values in this report for reasons presented in the Introduction (See Section 1.3.7).

2.3.3 The "100% Test" Protocol

The purpose of the "100% test" is to test samples at a concentration higher than that allowed by the Microtox Basic test (69). One of the disadvantages of this test is that it is more sensitive to operator technique (pipetting), and results may not be quite as precise (69).

The 100% test requires only five cuvettes to be used for each test, placed in wells A1 through A5. As per the Basic test, 1000 µL of Microtox Diluent is added into each cuvette in wells A1 through A4. Osmotic adjustment is performed as before by adding 250 µL of MOAS to the cuvette in well A5. Two thousand five hundred microliters of the sample is then added to this cuvette and mixed by filling and dispensing the pipettor 3 to 4 times. Then a two-fold dilution series is performed by first transferring 1000 µL from A5 to A4 and mixing with the pipettor (69). Two other 1000 µL transfers are performed (A4 to A3 and A3 to A2) and the resulting dilutions are mixed as before. Then 1000 µL is discarded from the cuvette in the A2 well and 750 µL is discarded from the cuvette in well A5 (69).

When all the dilutions are prepared, 10 µL of the Microtox Reagent is added to each cuvette. Upon the addition of the reconstituted Reagent to each cuvette, which have final concentrations of 45%, 22.5%, 11.25%, 5.625%, and 2.8%, a timer is started on the Microtox software. Then at predetermined intervals (e.g. 5 and 15 min) light readings are taken as

prompted by the computer software. As with the Basic test, once all the light readings have been taken, then the computer software generates an IC_{50} value (69).

2.4 Acute Lethality Tests using *Daphnia magna*

Work with *Daphnia magna* was carried out at Chemex Labs in Edmonton, AB under the supervision and guidance of Allen Verbeek. Throughout the testing period, a 10.0 g/L NaCl solution was used as a control. In all instances, the LC_{50} of this solution fell within acceptable guidelines.

One aspect of toxicity testing with *D. magna* as opposed to the Microtox system is the requirement of a maintenance culture (30). Information on the upkeep of this culture is beyond the scope of this report, but excellent protocols and information can be obtained in the Environment Canada Environmental Protection Series on *Daphnia* spp. testing. The following protocol is from this Environment Canada report.

Tests can be performed in an environmental chamber or cooler as long as the temperature is maintained at 20±2 °C. As a photoperiod is required, the facility must be either a separate laboratory or a portion of a laboratory enclosed by curtains. The photoperiod (16±1 h light: 8±1 h dark) is to be timed to coincide with that to which the organisms have been acclimated. Test vessels must be constructed of glass or clear plastic. Due to the possibility of some of the thiophenes adhering to plastic vessels, 250 mL glass beakers (Fisher Scientific) were used for all tests in this study.

Reconstituted water was prepared for the control water by adding the following amounts of chemicals to one liter of deionized water: 192 mg $NaHCO_3$; 120 mg $CaSO_4 \cdot 2H_2O$; 120 mg $MgSO_4$; 8 mg KCl. The reconstituted

water was aerated vigorously in a nontoxic vessel for at least 24 h prior to use, by passing oil-free compressed air through glass airstones (Cameron Management, Calgary, AB) and was adjusted to the test temperature prior to use. The control water had a dissolved oxygen content of 90 to 100% air saturation.

The test concentrations and numbers of test solutions prepared depend on the purpose of the test. For the majority of tests in this study, a total of five test concentrations were prepared for analysis. In some instances, as many as seven test concentrations were used for a single test.

Neonates that were less than 24-h-old were used in each test. Daily removal of neonates from the maintenance culture ensured a readily available supply of neonates for daily testing. Temperature, dissolved oxygen, conductivity and pH of the test solutions were checked before starting the test. Equal numbers of neonate daphnids at least ten in each treatment, were introduced into each test solution and the control water, without exceeding the loading density of no more than one individual per 15 mL of reconstituted water.

The transfer of daphnids to the test vessels was done as quickly as possible, with minimal addition of culture water and minimal stress to the organisms. The tip of the transfer pipette was held under the surface of the test solution while releasing the organisms and the neonates were inspected after transfer. Any which were floating or injured were replaced.

The test was a 48-h static test, that is there was no replacement of solutions during the test. The daphnids were not fed during the test nor were any of the test solutions aerated. The volume of each test solution was 200 mL. If there was a mortality greater than 10% in the control, then the test was considered invalid.

The duration of the 48-h test allows all daphnids to proceed through one molt, at which time they are most sensitive, but does not prolong the test to the point that starvation becomes a major factor. At each observation time, the number of dead daphnids in each test vessel was recorded, and those daphnids were removed (30). Death is indicated by lack of movement of the body, appendages, and heart as observed through a dissecting microscope. Some compounds may cause the daphnids to become completely immobile and the heart rate may slow to 1 to 2 beats/min In instances such as these, the heart beat becomes the final determining factor for death.

After 48 h, the mortalities were recorded in each test solution. The 48-h LC_{50} value and the corresponding 95% confidence limits were then calculated.

2.5 Microtox Testing of Supernatants of Cultures Grown in the Presence of Condensed Thiophenes

The toxicity tests described in Section 2.3 were limited to those compounds that could be purchased or chemically synthesized in high purity and sufficient quantity to use in the Microtox assay. However, many of the bacterial metabolites of benzothiophenes and dibenzothiophene are very difficult to synthesize. Among these are the sulfoxides of benzothiophene and the methylbenzothiophenes (32, 60, 82) and 3-hydroxy-2-formylbenzothiophene (HFBT) which is a common metabolite of dibenzothiophene (58, 71).

Because many of the condensed thiophenes are cometabolized by bacteria, the strategy used for these experiments was to grow bacterial cultures on a utilizable carbon source with one of the condensed thiophenes as a cometabolic substrate in a liquid medium. Then at various times,

supernatant samples were removed and assayed using the Microtox method to detect increases or decreases in IC_{50} values and allow screening of undetected and unidentified metabolites that might be present in the batch culture supernatant.

The experimental design required three controls, along with the test culture, to be assayed by the Microtox procedures at each sampling time. One was a sterile control containing only the growth substrate at the initial concentration used in the test cultures. This ensured that the growth substrate did not affect the activity of *P. phosphoreum* used as the test organism in the Microtox assay. A second sterile control containing the growth substrate and the condensed thiophene was required to ensure that the toxicity of the culture medium was not altered by abiotic processes, such as evaporative loss of the condensed thiophene. A third control, containing the growth substrate and the condensed thiophene-transforming bacterium, was required to determine if bacterial metabolism of the growth substrate changed the toxicity of the medium.

The initial experiments used *Pseudomonas* strains F and W1 with glucose (1.25 g/L) as the growth substrate, and benzothiophene or dibenzothiophene as the test compound. These cultures had been shown to oxidize a variety of benzothiophenes and dibenzothiophenes when grown on glucose (32, 60). However, the sterile control containing glucose showed that it stimulated the light emissions of *P. phosphoreum*. Thus, glucose could not be used as a growth substrate.

Five other possible growth substrates, (sucrose, xylose, fructose, galactose and glutamic acid, [Sigma Chemical Co.,St. Louis, MO]) were then evaluated for their use in these experiments. To be suitable, they must support the growth and oxidizing activities of the condensed thiophene-

transforming bacteria (*Pseudomonas* strains W1 and F), but not affect the activity of *P. phosphoreum*. At a concentration of 1.25 g/L, glutamic acid, (an amino acid) supported the growth of both strains, and did not inhibit or stimulate the light emissions of *P. phosphoreum*. Therefore, glutamic acid was used in subsequent studies.

The other important parameter to manipulate was the initial amount of the condensed thiophene in the growth culture. It had to be below the saturation concentration of the thiophene, so that the organosulfur compound was present in only one phase in the culture fluid. The saturation level had to be determined so that compounds were added below this level. If a compound was added to a culture system at a concentration above the saturation point, then the particulate chemical would continue diffusing into solution as the levels of the soluble compound decreased either through weathering or by microbial oxidation. This would result in a plateau level of toxicity as the excess diffused into solution. Once all of the chemical was in solution, then it would be expected that a steady decrease in toxicity would be observed as the culture degraded this compound assuming that the thiophene was being converted into a less toxic compound. Also, the concentration of the condensed thiophene had to be in the range of its IC_{50} value, so that any changes in the toxicity of the culture supernatant resulting from the activities of the thiophene-transforming bacteria could be detected by the Microtox test.

Pseudomonas isolates W1 and F were used in these experiments. Cultures were maintained on mineral medium (B+NP) which contained per liter 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 2.0 g Na_2SO_4, 2.0 g KNO_3, 0.2 $MgSO_4 \cdot 7H_2O$, trace amount $FeSO_4 \cdot 7H_2O$, 1 mL trace metals solution (32), supplemented with glutamic acid (Sigma Chemical Co., St. Louis, MO) at a concentration of

1.25 g/L. According to Saftic´ *et al.* (83) these isolates are motile, Gram-negative rods, oxidase positive, catalase positive, indole negative and methyl red negative. Isolate W1 produced a fluorescent yellow-green pigment on Pseudomonas Agar F (Difco Laboratories, Detroit, MI), whereas isolate F produced no pigment on this medium nor on Pseudomonas Agar P (83).

Thus, the conditions used for growing the condensed thiophene-transforming bacteria were as follows. An appropriate concentration of the thiophene was added to 200 mL B+NP medium supplemented with glutamic acid (1.25 g/L) in a sealed, screw-capped with a Teflon liner 500-mL flask to prevent evaporation of the condensed thiophene. After inoculation, the culture and the controls were incubated at room temperature with shaking at 100 rpm and samples were removed at various times for toxicity testing. When the flask was opened to remove samples for the Microtox test, air was admitted into the flask to replenish the supply of O_2.

2.5.1 Cultures Grown in the Presence of Benzothiophene

A saturated solution of benzothiophene was prepared in sterile B+NP medium containing glutamic acid. Then a portion of this solution was diluted (1:16) in the same medium to give an initial benzothiophene concentration of 9.8 mg/L. This was inoculated with *Pseudomonas* isolate F (5% by volume) that had been grown in B+NP medium containing glutamic acid in a sealed, screw-capped 500-mL flask to prevent evaporation of the benzothiophene. The inoculum was prepared by adding *Pseudomonas* isolate F (5% by volume) to B+NP medium containing glutamic acid. Inocula were placed on a rotary shaker at 250 rpm, 28°C for 24 h. This was repeated for each experiment.

2.5.2 Cultures Grown in the Presence of Dibenzothiophene

Dibenzothiophene was dissolved in methylene chloride and enough of this solution was added to two empty sterile 500-mL flasks so that each received 0.24 mg of dibenzothiophene. After the methylene chloride had evaporated, 200 mL of sterile B+NP medium was added to each flask and these were allowed to sit at room temperature for 1 h to allow the dibenzothiophene dissolve. One flask was inoculated with *Pseudomonas* isolate F (5% by volume) while the other remained uninoculated, and both were incubated along with two other controls devoid of dibenzothiophene.

2.5.3 Cultures Grown in the Presence of 3-Methylbenzothiophene

A saturated solution of 3-methylbenzothiophene was prepared in sterile B+NP medium containing glutamic in the same manner as for benzothiophene. A volume of this saturated solution was added to flasks to give an initial concentration of 4.0 mg/L. This flask was then inoculated with a 5% inoculum of *Pseudomonas* isolate F. Controls were used as mentioned above.

2.5.4 Cultures Grown in the Presence of 5-Methylbenzothiophene

As with benzothiophene and 3-methylbenzothiophene, a saturated solution of 5-methylbenzothiophene was prepared in sterile B+NP medium containing glutamic acid. 5-Methylbenzothiophene was added to give a final concentration of 4.5 mg/L. To this, a 5% inoculum of *Pseudomonas* isolate F was added to the test flask. Controls were used as mentioned above.

2.5.5 Toxicity of Supernatants When Grown on Condensed Thiophenes (Isolate W1)

This part of the study involved testing for detoxification of condensed thiophenes using the *Pseudomonas* isolate W1. The experiment was set up identically to the experiment with isolate F. Isolate W1 was maintained on the same medium as isolate F, as described in Section 2.5.

3 Results

3.1 Microtox Results with Methanol Solutions of Selected Organosulfur Compounds

Figures 1 and 2 are printouts generated from the Microtox system for benzothiophene and 3-methylbenzothiophene. The first part of the printouts includes information on the sample being tested such as the file name, description, the method used (Basic or 100% test), initial concentration, test time, the type of osmotic adjustment used, dilutions used, and concentration units (% or mg/L). The headings for tabulated columns of data and calculated results presented in the Microtox Report are as follows: the first column indicates the number of the sample dilution; the second column shows the raw light level data recorded during the test, the I_O (initial light output as recorded) and I_T (light output recorded at time T) readings for each numbered sample dilution; the third column shows each sample concentration, (expressed in the units of measurement selected); the fourth column shows the Gamma value calculated from the data.

A correction factor is added to compensate for the effects of light drift (up or down), the offset in light output due to the dilution which occurs when the organisms are challenged, and reagent pipetting errors. The report includes a graph plotting the concentration of the sample (dose) against its effects on the test organisms. On a log-log scale, the plot of concentration against Gamma is usually a straight line. This graph allows for further analysis of the data. If a point is far from the dose-response curve, then it should be treated with suspicion.

At the bottom of the printout, the EC_{50} value is given. This is accompanied by 95% Confidence Range. This range is also represented by the horizontal lines that make the dose response curve into a band, rather

MICROTOX DATA REPORT

FILE NAME: BT#3-0.K5

TEST DATE:_____
TEST TIME:_____

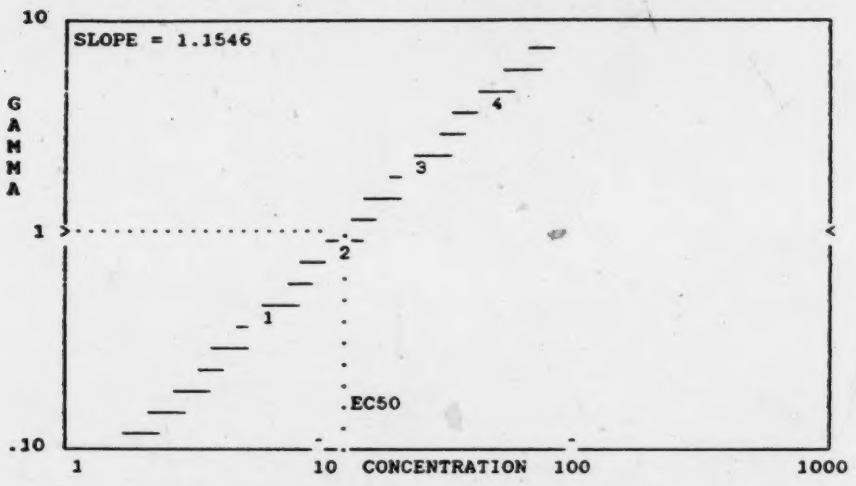
Sample Description:
BT#3 TIME 0 THURSDAY MARCH 30/95

Procedure: BASIC
Initial Concentration : 45 %
Test Time: 5 minutes

Osmotic Adjustment: MOAS
Dilution Factor : 2
Concentration Units: %

NUMBER	IO/IT	CONC.	GAMMA
1	81/ 50	5.6250	0.41700#
2	80/ 36	11.2500	0.94359#
3	84/ 24	22.5000	2.09297#
4	85/ 13	45.0000	4.60619#

CONTROL IT/IO = 72/ 82 CORRECTION FACTOR = 0.8784



EC50 11.9207 (95% CONFIDENCE RANGE: 11.6848 TO 12.1613)

Used for calculations

Figure 1. Microtox Data Report for a solution of benzothiophene prepared in methanol.

MICROTOX DATA REPORT

FILE NAME:

TEST DATE:_____
TEST TIME:_____

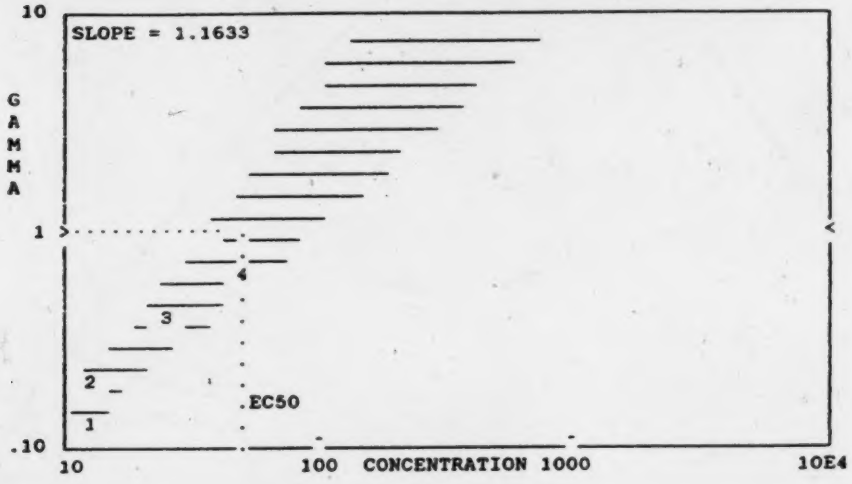
Sample Description:

Procedure: BASIC
Initial Concentration : 45 %
Test Time: 5 minutes

Osmotic Adjustment: MOAS
Dilution Factor : 2
Concentration Units: %

NUMBER	IO/IT	CONC.	GAMMA
1	84/ 71	5.6250	0.06448#
2	87/ 64	11.2500	0.21109#
3	86/ 55	22.5000	0.41123#
4	85/ 43	45.0000	0.75885#

CONTROL IT/IO = 78/ 87 CORRECTION FACTOR = 0.8937



EC50 49.8007 (95% CONFIDENCE RANGE: 24.5673 TO 100.9519)

Used for calculations

Figure 2. Microtox Data Report for a solution of 3-methylbenzothiophene prepared in methanol.

than a simple line. The shorter the horizontal band, the better the data consistency.

Knowing the concentration of the test solution (in mg/L or μM), the EC_{50} can be calculated in those respective units from the percentage by volume data given in Fig. 1.

The results from the initial toxicity testing of several organosulfur compounds dissolved in methanol are presented in Table 1. Most entries for the oxidized compounds have IC_{50} values that are greater than the test concentrations. In these cases, no toxicity was observed in the Microtox test. Extrapolation can give some very wide confidence intervals. For example, one of the determinations with dibenzothiophene sulfone in methanol (Table 1) gave an IC_{50} value of 18 mg/L, with a 95% confidence interval of 1.2 to 280 mg/L. Ideally, the IC_{50} should be obtained by interpolation rather than extrapolation.

Only the tests with 3-methylbenzothiophene yielded IC_{50} values obtained by interpolation. The 3-methylbenzothiophene solution was prepared by adding 100 μL of this compound to 1 L of methanol. Because the density of this compound was not known, it was assumed to be 1.18 g/mL, which is the density of benzothiophene (95). The IC_{50} values from the 5- and 15-min tests for 3-methylbenzothiophene were 0.38 and 0.41 mg/L, respectively. Difficulties were experienced getting sufficiently high concentrations of the organosulfur compounds dissolved in methanol for subsequent toxicity testing.

Table 1 Results from the Microtox assay using solutions of organosulfur compounds prepared in methanol prior to diluting in the bioassay test solutions.

Compound	Stock Solution Concentration (mg/L)	5-min Test IC_{50} (mg/L)	5-min Test 95% C.I. (mg/L)	15-min Test IC_{50} (mg/L)	15-min Test 95% C.I. (mg/L)
Benzothiophene	272	1.6[a]	0.95 - 2.7	1.6[a]	1.2 - 2.3
Benzothiophene sulfone	374	>1.8[b]	N/A[c]	7.4[a]	1.8 - 30
3-Methylbenzo-thiophene[d]	118	0.38	0.33 - 0.44	0.41	0.34 - 0.50
Dibenzothiophene	31	0.15[a]	0.13 - 0.18	0.16[a]	0.12 - 0.22
Dibenzothiophene sulfoxide[e]	172	>0.85	N/A	>0.85	N/A
Dibenzothiophene sulfone	273	>1.4	N/A	18[a]	1.2 - 280

[a]Extrapolated value of IC_{50} which was greater than the lowest dilution tested.
[b]No toxicity observed, thus the IC_{50} is greater than the maximum concentration tested and given.
[c]Not applicable because no value for IC_{50} was determined.
[d]Concentration based on an assumed density of 1.18 g/mL.
[e]Commercially available preparation used.

3.2 Microtox Results with DMSO Solutions of Selected Organosulfur Compounds

DMSO proved to be a better solvent for the OSC than methanol, allowing solutions with higher concentrations of these compounds to be prepared. The main objective of this work was to test the reproducibility of the results obtained with the Microtox method. Individual solutions of each compound were prepared in DMSO, and these were used twice to determine the IC_{50} values. Then a second series of fresh solutions was prepared and the toxicity tests were done with these solutions for a total of three tests for each compound.

Among all of the compounds tested (Table 2), the IC_{50} values for dibenzothiophene sulfone were the only ones obtained by extrapolation. The IC_{50} values for benzothiophene were very reproducible, and ranged from 1.2 to 1.6 mg/L. Each value from the second solution was within the 95% confidence interval of the other assays. Similarly, the IC_{50} values for benzothiophene sulfone were reproducible, ranging from 6.2 to 6.7 mg/L, with each of these values falling within the 95% confidence interval of the other two assays.

The dibenzothiophene IC_{50} values were the lowest observed in Table 2, indicating that it had the highest toxicity among the compounds tested. The IC_{50} values ranged from 0.18 to 0.25 mg/L. From the 5 and 15 min test with dibenzothiophene sulfoxide, there was good agreement among the IC_{50} values, ranging from 20 to 24 mg/L.

Although the IC_{50} values for dibenzothiophene sulfone were extrapolated, they were in good agreement, ranging from 15 to 21 mg/L. When a saturated solution of dibenzothiophene sulfone in DMSO was used for the Microtox test, the IC_{50} value could only be determined by

Table 2 Results from the Microtox assays using solutions of organosulfur compounds prepared in DMSO prior to diluting in the bioassay test solutions.

Compound	Stock Solution Concentration No.	Stock Solution Concentration (mg/L)	5-min Test IC_{50} (mg/L)	5-min Test 95% C.I. (mg/L)	15-min Test IC_{50} (mg/L)	15-min Test 95% C.I. (mg/L)
Benzothiophene	1	360	1.2	0.9 - 1.6	1.3	1.0 - 1.7
	1	360	1.5	1.4 - 1.7	1.6	1.5 - 1.7
	2	372	1.1	1.1 - 1.2	1.2	1.0 - 1.3
Benzothiophene sulfone	1	452	9.1	7.7 - 10.7	6.2	5.3 - 7.3
	1	452	9.0	7.9 - 10.2	6.2	6.0 - 6.5
	2	453	9.7	8.4 - 11.1	6.7	6.1 - 7.3
3-Methylbenzo-thiophene[a]	1	118	0.59	0.50-0.71	0.72	0.65- 0.80
	1	118	0.61	0.55-0.67	0.64	0.56 - 0.74
	2	118	0.69	0.64- 0.74	0.76	0.67 - 0.85
3-Methylbenzo-thiophene sulfone	1	562	0.34	0.31- 0.37	0.38	0.35 - 0.41
	1	562	0.59	0.58-0.59	0.63	0.61-0.64
Dibenzothiophene	1	31	0.18	0.17- 0.19	0.18	0.17- 0.19
	1	31	0.16	0.14- 0.18	0.18	0.14 - 0.22
	2	31	0.23	0.21- 0.25	0.25	0.22 - 0.28
Dibenzothiophene sulfoxide[b]	1	757	20	18 - 22	20	17- 24
	1	757	23	21- 26	21	19 - 24
	2	761	24	19 - 29	23	20 - 26
Dibenzothiophene sulfone	1	277	>21[c]	12.0 - 34	>21[c]	14 - 30
	1	277	>22[c]	6.0 - 78	>20[c]	2.1 - 190
	2	280	>17[c]	6.0 - 50	>15[c]	12- 18

[a]Concentration based on an assumed density of 1.18 g/mL.

[b]Commercial preparation

[c]Extrapolated value of IC_{50} which was greater than the lowest dilution tested.

extrapolation. Thus, a more accurate IC_{50} value could not be determined. Dibenzothiophene sulfone may be least toxic of the compounds tested with Microtox.

The 15-min assays showed that 3-methylbenzothiophene had IC_{50} values ranging from 0.64 to 0.76 mg/L. Replicate tests with 3-methylbenzothiophene sulfone showed IC_{50} values of 0.38 and 0.63 mg/L in the 15-min test .

The results in Table 2 indicate that for a given compound, there was little difference between the IC_{50} values determined in the 5-min and 15-min tests. For example, for benzothiophene the 5-min and 15-min IC_{50} values (in mg/L) from the three tests were: 1.2 and 1.3; 1.5 and 1.6; 1.1 and 1.2, respectively. Similarly, from the values for dibenzothiophene, IC_{50} values (in mg/L) from the 5-min and 15-min tests were: 0.18 and 0.18; 0.16 and 0.18; 0.23 and 0.25, respectively. These results suggest that the 5-min test may be adequate for determining the IC_{50} values of these compounds as these compounds do not seem to be mass transfer limited.

In Table 3, the molar concentrations were derived from the 15-min IC_{50} values from the Microtox tests with stock solutions no. 2 (Table 2), except for 3-methylbenzothiophene sulfone in which the lower IC_{50} values was used. The data in Table 3 show that the oxidized compounds are not markedly more toxic than the parent thiophenes. Only the sulfone of 3-methyl-benzothiophene is more toxic than its parent compound. The IC_{50} value for this sulfone (2.1 μM) is only slightly less than that of 3-methylbenzothiophene (5.1 μM).

In the other cases, the oxidized compounds are less toxic (have higher IC_{50} values) than their parent compound. For example, the IC_{50} for benzothiophene sulfone was found to be 38 μM, whereas that of

Table 3. Comparison of the IC_{50} values based on molar concentrations of the organosulfur compounds in stock solutions of DMSO. Data from the 15 min tests with solutions no. 2 shown in Table 2.

Compound	Molecular weight	Stock solution concentration (mg/L)	Stock solution concentration (mM)	IC_{50} (µM)	95% C.I. (µM)
Benzothiophene	134	372	2.8	9.0	7.5 - 9.7
Benzothiophene sulfone	166	453	2.	38	23 - 60
3-Methylbenzo-thiophene	148	118	0.8	5.1	4.5 - 5.7
3-Methylbenzo-thiophene sulfone	180	562	3.1	2.1	1.9 - 2.3
Dibenzothiophene	184	31	0.2	1.3	1.2 - 1.5
Dibenzothiophene sulfoxide[a]	200	761	3.8	115	99.9 - 132
Dibenzothiophene sulfone	216	280	1.3	>68[b]	56 - 84

[a]Commercial preparation

[b]Extrapolated value of IC_{50} which was greater than the lowest dilution tested.

benzothiophene was found to be 9 μM. This is a 4.2-fold difference. Similarly, the IC_{50} values for dibenzothiophene sulfoxide and sulfone were found to be 115 μM and >68 μM, respectively, whereas the IC_{50} for dibenzothiophene was found to be 1.3 μM. These differences are 88-fold and >52-fold, respectively.

3.3 HPLC Analysis of Organosulfur and Aromatic Compounds

From the standard curves of each of the compounds tested, the aqueous solubilities were determined and compared to published literature values. According to OECD guidelines, there is no one single method available to cover the whole range of solubilities in water (74). Two methods are outlined in the OECD guideline, the "column elution method", and the "flask method" (74). The column elution method is generally recommended for substances with low solubilities (74). This column method is based on the elution of a test substance with water from a micro-column which is charged with an inert carrier material such as glass beads, silica gel or sand and an excess of test substance (74). The water solubility is determined when the mass concentration of the eluate is constant (74). Even though the method employed in this study was different from that suggested by OECD guidelines, the results were comparable to published values.

The first chemicals tested were ones with published aqueous solubilities values. The reported solubilities for phenanthrene, naphthalene, and biphenyl are 1.29, 31 and 7 mg/L, respectively (65). From HPLC analysis, aqueous solubilities calculated here were similar to those of the published values (Table 4).

Table 4. Aqueous solubilities of selected aromatic compounds determined by HPLC. Comparison of solubilities determined using various methods to produce a supernatant free of undissolved test compound.

	Average concentration (standard deviation)[a], mg/L		
	Samples allowed to	Samples centrifuged in	
Compound	settle in flask	Plastic tube	Glass tube
Benzothiophene	150 (4.4)	106[b] (10)	67.3[b] (0.9)
	162 (3.7)	123[b] (11.5)	88.9[b] (4.0)
Benzothiophene	551 (6.5)	571[b] (37)	304[b] (4.1)
sulfone	580 (7.1)	ND[c]	ND
	500 (6.3)	ND	ND
3-Methylbenzothiophene	48.8 (1.7)	29.6[b] (3.0)	30.2[b] (0.6)
	49.1 (0.2)	32.3[b] (3.2)	31.8[b] (0.2)
3-Methylbenzothiophene	422 (8.6)	436 (14.7)	298[b](7.3)
sulfone	397 (2.1)	ND	ND
	345 (9.7)	ND	ND
5-Methylbenzothiophene	51.7 (0.5)	ND	ND
	40.7 (0.6)	ND	ND
5-Methylbenzothiophene	392 (4.1)	ND	ND
sulfone	405 (2.6)	ND	ND
Dibenzothiophene	1.2 (0.04)	0.5[b] (0.08)	1.0[b] (0.004)
	0.9 (0.15)	ND	ND
Dibenzothiophene	112 (0.3)[d]	107[b] (0.3)	111 (1.5)
sulfoxide	324 (1.0)	ND	ND
	326 (5.2)	ND	ND
Dibenzothiophene	5.8 (0.1)	5.8[b] (0.1)	2.0[b](0.1)
sulfone	5.75 (0.02)	5.62[b] (0.03)	5.69[b] (0.04)
Naphthalene	32.1 (0.7)	22.8[b] (1.3)	24.5[b] (0.4)
Biphenyl	6.9 (0.7)	2.4[b] (0.3)	5.0[b](0.2)
Phenanthrene	1.2 (0.1)	0.4[b] (0.2)	1.2 (0.1)
Acenaphthene	4.01 (0.3)	ND	ND

[a]Based on triplicate analyses.

[b]This mean is significantly less than the mean obtained from the "Sample allowed to settle in flask" based on the Duncan's multiple range test ($P<0.05$).

[c]ND - Not Done

[d]Commercial preparation

In most instances, especially with the chemicals having known solubilities, the samples that were drawn out of the flask that had been allowed to sit for an hour provided the most accurate results. Also with the thiophenes examined, the samples drawn out of the flask gave the highest aqueous solubility value.

From HPLC analysis, the sample taken from the flask that was allowed to settle had a biphenyl concentration of 6.9 mg/L (reported value = 7 mg/L (65)). The plastic centrifuged sample had a concentration of 2.4 mg/L while the glass centrifuged sample had a concentration of 5.0 mg/L. With naphthalene the settled sample had a concentration of 32.1 mg/L which compared favorably to the reported value of 31 mg/L (65). The plastic centrifuged sample had a concentration determined to be 22.8 mg/L while the glass centrifuged sample had a concentration of 24.5 mg/L. Phenanthrene concentration for the settled sample was 1.15 mg/L which compared well with the reported value of 1.29 mg/L (65). The plastic centrifuged sample had a concentration determined to be 0.44 mg/L while the glass centrifuged sample had a concentration of 1.21 mg/L.

From these results it was concluded that the best method for determining aqueous solubilities was from the settled samples. Comparison of the settled values to the reported values show our results to be consistent with the reported values except in the case of phenanthrene in which the glass centrifuged sample provided the concentration closest to that of the reported literature values.

As a final evaluation of the reliability of the HPLC method, the analyst was given a sample of acenaphthene in a "blind test". The solubility of this compound was determined to be 4.01 ± 0.3 mg/L. MacKay and Shiu (65) reported the solubility of acenaphthene to be 3.93 ± 0.01 mg/L. Clearly, the

HPLC method yielded reliable results. The standard curve for acenaphthene, which was typical of the other standard curves, is presented in Figure 3.

Vassilaros *et al.* (94) reported solubility values for benzothiophene and dibenzothiophene of 113 and 1.7 mg/L respectively. By comparison, the mean value in Table 4 for benzothiophene was 156 mg/L and for dibenzothiophene 1.05 mg/L.

From Table 4 it is clear that the oxidized derivatives of the OSC are more soluble than the parent compounds which would be expected from increasing their polarity. On a mass basis benzothiophene sulfone was 3.4-times more soluble than benzothiophene. The sulfoxide of dibenzothiophene was 310-times more soluble than the parent compound while the sulfone of dibenzothiophene was only 5.5-times more soluble than dibenzothiophene. The same trend follows for the methylated benzothiophenes used in this experiment. The sulfones of 3-methyl and 5-methylbenzothiophene were 7.9 and 8.6-times respectively more soluble than their parent compounds. These results would be expected as the oxidation of a compound would make it more polar and subsequently more soluble than the parent compound. It would be expected that other oxidized derivatieves of these compounds would be more soluble than the parent compounds.

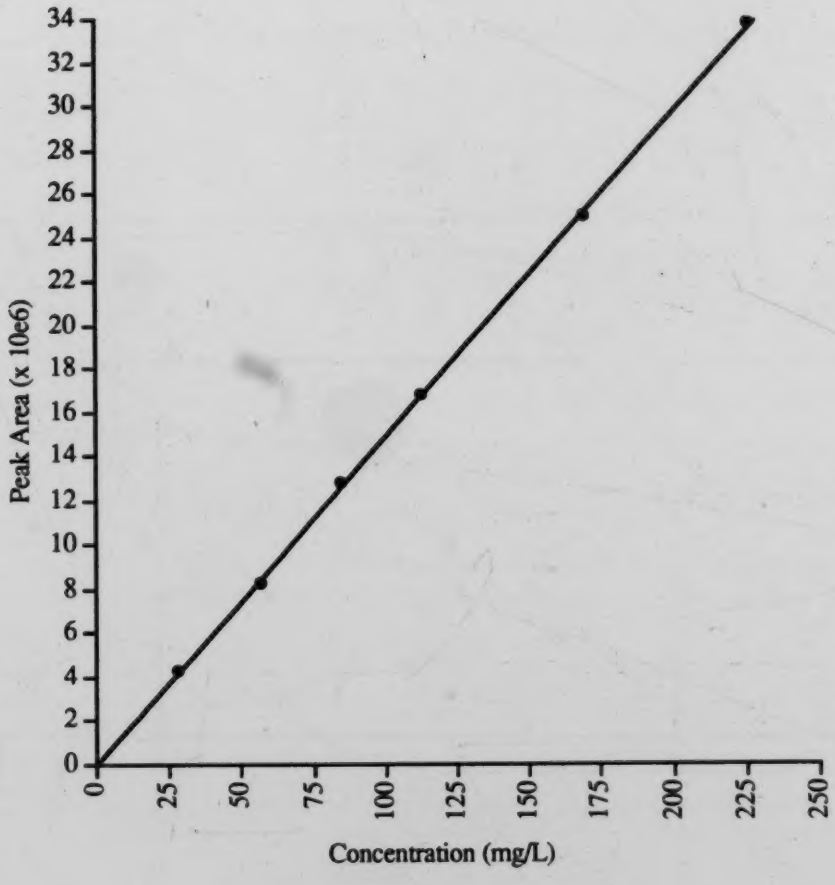


Figure 3. Calibration curve for acenaphthene from HPLC analyses. This curve is typical of all other graphs prepared for determining aqueous solubilities.

3.4 Microtox Results with Aqueous Solutions of Selected Organosulfur Compounds

Saturated solutions of the test compounds were prepared and their concentrations were determined by HPLC (Table 4). Appropriate dilutions of these solutions were prepared and used for the Microtox tests, and the results are given in Table 5. Microtox results based on molar concentrations are presented in Table 6. In general, there was good agreement between the replicate IC_{50} values for those compounds that gave reproducible saturation concentrations (e.g. benzothiophene and 3-methylbenzothiophene). In contrast, the replicate IC_{50} values differed for some compounds which gave markedly different saturation concentrations (e.g. benzothiophene sulfone and 3-methylbenzothiophene sulfone). Benzothiophene-2,3-dione and 5-methylbenzothiophene-2,3-dione had good agreement in their aqueous solubilities yet their IC_{50} values were markedly different.

In most cases, there was good agreement between these IC_{50} values determined from the saturated aqueous solutions (Table 5) and those determined from the DMSO solutions (Table 2). For example, work with the saturated aqueous solution gave an IC_{50} of 2.0 mg/L for benzothiophene, whereas work with the DMSO solutions gave values between 1.3 and 1.6 mg/L for this compound. The closest agreement between the two methods was for dibenzothiophene. The saturated aqueous solutions gave IC_{50} values of 0.12 and 0.19 mg/L (Table 5), whereas the DMSO solutions gave values between 0.18 and 0.25 mg/L (Table 2). The worst agreement was for 3-methylbenzothiophene sulfone. The saturated aqueous solutions gave IC_{50} values of 23 and 36 mg/L (Table 5), whereas the DMSO solutions gave values of 0.38 and 0.63 mg/L (Table 2).

Table 5 Results from Microtox tests using dilutions of saturated aqueous solutions of the organosulfur compounds.

Compound	Stock Solution Concentration (mg/L)	5 min Test IC_{50} (mg/L)	5 min Test 95% C.I. (mg/L)	15 min Test IC_{50} (mg/L)	15 min Test 95% C.I. (mg/L)
Benzothiophene	156	1.9	1.8 - 2.0	2.0	1.97 - 2.02
	162	1.4	1.3 - 1.5	1.4	1.3 - 1.5
Benzothiophene sulfone	500	7.1	6.7 - 7.4	5.7	5.4 - 6.1
	551	13.6	13.2 - 13.8	10.2	9.7 - 10.6
	580	6.7	5.9 - 7.5	4.9	4.6 - 5.3
Benzothiophene-2,3-dione[a]	405	275	216-351	279	68 -1134
	412	87	66 - 114	107	91 - 125
3-Methylbenzo-thiophene	48.8	0.82	0.70 - 0.95	0.84	0.70 - 1.0
	49.1	0.48	0.34 - 0.67	0.50	0.36 - 0.67
3-Methylbenzo-thiophene sulfone	345	31.6	28.6 - 34.8	31.6	27.6 - 36.2
	397	23.4	22.1 - 24.9	23.4	21.8 - 25.2
	422	35.7	34.1 - 37.4	34.4	33.4 - 35.5
5-Methylbenzo-thiophene	51.7	0.72	0.69 - 0.75	0.68	0.62 - 0.74
	40.1	0.72	0.63 - 0.83	0.72	0.63 - 0.84
5-Methylbenzo-thiophene sulfone	405	26.5	17.3 -40.7	28.2	19.8 - 40.1
	364	33.2	30.6 - 36.2	38.1	35.1 - 41.4
	392	79	58 - 107	91	72 - 114
5-Methylbenzo-thiophene-2,3-dione[a]	416	150	123-182	124	97.2-164
	422	48	41 - 57	57	49 - 67
7-Methylbenzo-thiophene-2,3-dione[a]	426	80.1	77.1-83.5	93.7	76.7-114
Dibenzothiophene	1.2	0.19	0.16 - 0.22	0.20	0.18 - 0.22
	0.9	0.12	0.11 - 0.12	0.12	0.11 - 0.14
Dibenzothiophene sulfoxide	324	4.9	3.9 - 6.2	3.4	2.4 - 4.9
	326	1.3	1.0 - 1.7	0.8	0.6 - 1.1
Dibenzothiophene sulfone	5.8	>5.8[b]	N/A[c]	>5.8[b]	N/A[c]
	5.7	>5.7[b]	N/A[c]	>5.7[b]	N/A[c]
Phenol	100	25	21 - 28	N/D[d]	

[a]At the neutral pH of the toxicity test, the 2,3-diones exist as very soluble 2-mercaptophenylglyoxalates (27). Thus, these compounds were prepared to the concentrations stated.

[b]No toxicity observed, thus the IC_{50} is greater than the maximum concentration tested and given.

[c]Not applicable because no value for IC_{50} was determined.

[d]Not Done. A 15-min test is not performed on phenol as the toxic effect is at its maximum at 5-min (69).

Table 6 Comparison of the IC_{50} values from the Microtox test based on molar concentrations of the organosulfur compounds in aqueous solution. Data from the 15-min tests.

Compound	Molecular weight	Stock solution concentration (mg/L)	(mM)	IC_{50} (µM)	95% C.I. (µM)
Benzothiophene	134	156	1.2	14.7	14.3 - 15.1
		162	1.2	10.4	9.9 -10.9
Benzothiophene sulfone	166	500	3.0	34.3	32.5 - 36.7
		551	3.3	63.7	58.4 - 63.9
		580	3.5	29.5	27.7 - 31.9
Benzothiophene-2,3-dione	266	392	1.5	342	271 - 429
		405	1.5	1049	257-4263
3-Methylbenzothiophene	148	48.8	0.3	5.7	4.7 - 6.8
		49.1	0.3	3.3	2.4 -4.6
3-Methylbenzothiophene sulfone	180	345	1.9	175	153 - 201
		397	2.2	130	121 - 140
		422	2.3	191	185 - 197
5-Methylbenzothiophene	148	51.7	0.35	4.6	4.2 - 5.0
		40.1	0.27	4.9	4.3 - 5.7
5-Methylbenzothiophene sulfone	180	405	2.3	157	110 - 223
		364	2.0	212	195 - 228
5-Methylbenzothiophene-2,3-dione	277	412	1.5	386	329 - 451
		416	1.5	448	351-592
7-Methylbenzothiophene-2,3-dione	277	422	1.5	206	177 - 242
		426	1.5	338	277-412
Dibenzothiophene	184	1.2	0.0065	1.1	1.0 - 1.2
		0.9	0.0049	0.7	0.6 - 0.8
Dibenzothiophene sulfoxide	200	324	1.6	17.0	12.0 - 24.5
		326	1.6	4.0	3.0 - 5.5
Dibenzothiophene sulfone	216	5.8	0.03	>27[a]	N/A[b]
		5.7	0.03	>27[a]	N/A[b]
Phenol[c]	94	100	1.1	261	225 - 303

[a]No toxicity observed, thus the IC_{50} is greater than the maximum concentration tested and given.
[b]Not applicable because no value for IC_{50} was determined.
[c]Phenol results calculated with 5-min data.

Dibenzothiophene sulfone was not very toxic (Table 5), and its limited aqueous solubility prevented the determination of its IC_{50}. Determining the IC_{50} value for dibenzothiophene sulfone using DMSO solutions was also hampered by its low solubility in DMSO and formation of a precipitate, leading to extrapolated values given in Table 2.

Also shown in Table 5 are the IC_{50} values for three 2,3-diones. Because of their high solubility, there was no need to prepare saturated solutions of these compounds. Known amounts of the 2,3-diones were weighed into volumetric flasks and dissolved in B+NP medium (pH 7). These compounds had the highest IC_{50} values among the organosulfur compounds tested (Table 5).

The IC_{50} value for phenol was also included in Tables 5 and 6. Phenol is used as a reference toxicant in the Microtox test (69). By comparing the OSC to phenol, a guideline can be established as to how toxic these compounds can be. A 15-min test is not performed for phenol because it produces its most toxic effects at 5 min. Based on the IC_{50} values, many of the thiophenes tested are more toxic than phenol.

The data obtained from dilutions of saturated aqueous solutions showed similar trends to the results obtained from DMSO solutions (Table 6). For example, there was a 3.4-fold difference between the IC_{50} values for benzothiophene and its sulfone, with the latter having the higher IC_{50}. Also, the oxidized dibenzothiophenes were less toxic than dibenzothiophene. The IC_{50} value for the sulfoxide was 12-times higher than that of dibenzothiophene and the IC_{50} value for the sulfone was >30-times greater than that of dibenzothiophene.

3-Methylbenzothiophene sulfone gave an IC_{50} value that was 37-fold greater than that of 3-methylbenzothiophene. 5-Methylbenzothiophene

sulfone gave an IC_{50} value that was 39-fold greater than that of 5-methylbenzothiophene (Table 6). This result was markedly different from the results obtained with the DMSO solutions (Table 2) for which the IC_{50} value for the sulfone was slightly less than that of 3-methylbenzothiophene. The molar IC_{50} values for three 2,3-diones are also shown in Table 6. The IC_{50} value of benzothiophene-2,3-dione was at least 23-fold greater than that of benzothiophene, and the IC_{50} value of 5-methylbenzothiophene-2,3-dione was 88-fold greater than that of 5-methylbenzothiophene. The toxicity of 7-methylbenzothiophene-2,3-dione was slightly higher than the toxicities of the other two diones. 7-Methylbenzothiophene was not available for these studies. The data in Table 6 clearly show that the oxidized compounds which have been identified as microbial metabolites of selected condensed thiophene are less toxic, on a molar basis, than the parent thiophenes.

As a quality control step, phenol is used at the beginning of each series of Microtox tests to ensure that the procedure provides acceptable results. A typical IC_{50} value for phenol is included in Table 5, against which the IC_{50} values of the organosulfur compounds can be compared. Microbics recommends that a well-performed test for phenol should be between 13 and 26 mg/L. This variation takes into account the natural variation that can occur in a typical test.

The parent compounds (benzothiophene, 3-methyl-benzothiophene, 5-methylbenzothiophene and dibenzothiophene) are all considerably more toxic than phenol. Benzothiophene sulfone and 3-methylbenzothiophene sulfone were considerably more toxic than phenol. Of the diones tested, only 7-methylbenzothiophene-2,3-dione was more toxic in one trial than phenol, while in another trial it was less toxic than phenol. It is reasonable to assume

that other alky-isomers of benzothiophene and dibenzothiophene would be more toxic than phenol.

3.5 *D. magna* Toxicity Testing with Aqueous Solutions of Selected Organosulfur Compounds

Saturated aqueous solutions of select OSC were prepared using reconstituted water. Due to the high solubility of benzothiophene-2,3-dione, 5-methylbenzothiophene-2,3-dione, 7-methylbenzothiophene-2,3-dione, and phenol these compounds were prepared to specified concentrations. The solutions were diluted to various concentrations for the *D. magna* test. The results are presented in Table 7. The LC_{50} values based on molar concentrations are presented in Table 8.

A computer generated output for determining the LC_{50} value is presented in Figure 4. The first part of the output has a table which gives the details of the test. The concentration of the sample is expressed as a percentage (v/v). The next two columns contain the number of organisms exposed to each concentration and the resulting number of mortalities. The number of dead is then given as a percentage. A value for LC_{50} is then given based on the moving average and the probit method. In this study the LC_{50} values were calculated based on the probit method.

Benzothiophene was approximately 9-times less toxic than the corresponding sulfone in the *Daphnia* test. There was no toxic effect observed with either dibenzothiophene or dibenzothiophene sulfone. The only derivative of dibenzothiophene to show any toxic effect in the *Daphnia* test was dibenzothiophene sulfoxide which had an average LC_{50} value of 8.3 mg/L.

Table 7 Results from *D. magna* toxicity test using dilutions of solutions of the organosulfur compounds

Compound	Stock Solution (mg/L)	LC_{50} as (%v/v) of Stock Solution	LC_{50}(mg/L)[a]	95% C.I.
Benzothiophene	158	30	47.0	39.9 - 53.5
	127	44	55.7	48.7 - 62.9
Benzothiophene sulfone	315	2.1	6.76	5.15 - 7.92
	157	3.1	4.84	4.28 - 5.51
Benzothiophene-2,3-dione	241	52	126	102 - 152
	190	84	160	138 - 196
3-Methylbenzothiophene	43.9	8.4	3.69	1.80 - 5.58
	44.8	2.4	1.06	0.14 - 2.84
3-Methylbenzothiophene sulfone	129		N/T[b]	
	143		N/T	
5-Methylbenzothiophene	59.4	38	22.3	17.1 - 27.6
	59.7	26	15.3	12.7 - 17.9
5-Methylbenzothiophene sulfone	178		N/T	
	163		N/T	
5-Methylbenzothiophene-2,3-dione	184		N/T	
	185		N/T	
7-Methylbenzothiophene-2,3-dione	186	1.5	2.70	1.55 - 4.19
	180	4.5	8.11	5.83 - 10.8
Dibenzothiophene	2.7		N/T	
	3.0		N/T	
Dibenzothiophene sulfoxide	116	9.4	10.9	7.44 - 14.9
	142	4.0	5.70	2.88 - 9.22
Dibenzothiophene sulfone	6.6		N/T	
	5.9		N/T	
Phenol	28.4	29	8.34	5.81 - 12.5
	27.1	31	8.37	6.31 - 11.1

[a] LC_{50} (mg/L) = LC_{50} x stock solution (mg/L) x (1/100)

[b] Sample was determined to be non-toxic.

Table 8 Comparison of the *Daphnia* LC_{50} values based on molar concentrations of the organosulfur compounds in aqueous solution.

Compound	Molecular weight	Stock solution concentration (mg/L)	(mM)	LC_{50} (μM)	95% C.I. (μM)
Benzothiophene	134	158	1.18	351	298 - 399
		127	0.95	416	363 - 469
Benzothiophene sulfone	166	315	1.90	40.7	31.0 - 47.7
		157	0.95	29.2	25.8 - 33.2
Benzothiophene-2,3-dione	266	241	0.91	474	383 - 571
		190	0.71	602	519 - 737
3-Methylbenzothiophene	148	43.9	0.30	24.9	12.2 - 37.7
		44.8	0.30	7.16	0.95 - 1.92
3-Methylbenzothiophene sulfone	180	129	0.72	N/T[a]	
		143	0.79	N/T	
5-Methylbenzothiophene	148	59.4	0.40	151	116 - 186
		59.7	0.40	103	85.8 - 121
5-Methylbenzothiophene sulfone	180	178	0.99	N/T	
		163	0.91	N/T	
5-Methylbenzothiophene-2,3-dione	277	184	0.66	N/T	
		185	0.67	N/T	
7-Methylbenzothiophene-2,3-dione	277	186	0.67	9.74	5.60 - 15.1
		180	0.65	29.3	21.0 - 39.0
Dibenzothiophene	184	2.7	0.01	N/T	
		3.0	0.02	N/T	
Dibenzothiophene sulfoxide	200	116	0.58	54.5	37.2 - 74.5
		142	0.71	28.5	14.4 - 46.1
Dibenzothiophene sulfone	216	6.6	0.03	N/T	
		5.9	0.03	N/T	
Phenol	94	28.4	0.30	88.7	61.8 - 133
		27.1	0.29	89.0	67.1 - 118

[a] Non-toxic, thus the LC_{50} is greater than the maximum concentration tested.

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**************************************************************************
CONC.          NUMBER          NUMBER          PERCENT          BINOMIAL
               EXPOSED         DEAD            DEAD             PROB.(PERCENT)
 15              10              10             100.0            0.09766
 10              10              10             100.0            0.09766
 5               10               4              40.0           37.69530
 2.5             10               2              20.0             46875
 1               10               0               0.0            .09766
 .25             10               0               0.0            0.09766

THE BINOMIAL TEST SHOWS THAT  1  AND  10  CAN BE
USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT
CONFIDENCE LIMITS BECAUSE THE ACTUAL CONFIDENCE LEVEL
ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.


AN APPROXIMATE LC50 OF  5.459357  IS OBTAINED BY
NONLINEAR INTERPOLATION BETWEEN  5  AND  10


----RESULTS CALCULATED USING THE MOVING AVERAGE METHOD---
SPAN          G               LC50            95 PERCENT CONFIDENCE LIMITS
 5             8.016342E-02                    3.511722       2.454491
 4.98196
 4             9.857717E-02                    4.046396       2.893588
 5.381692
 3             .1131913        4.321899        3.250424       6.037201
 2             .214883         4.711502        3.379551       6.358158

AN LC50 CALCULATED USING THE MOVING AVERAGE METHOD MAY NOT
BE A VERY GOOD ESTIMATE IF THE SPAN IS MUCH LESS THAN THE
NUMBER OF CONCENTRATIONS.


----RESULTS CALCULATED USING THE PROBIT METHOD-----------
ITERATIONS    G               H               CHI-SQUARE     PROBABILITY
 6             .2388562        1               2.698478       .609481

SLOPE  =       4.648715
95 PERCENT CONFIDENCE LIMITS = 2.376752    AND    6.920677

LC50  =        4.512599
95 PERCENT CONFIDENCE LIMITS  =   3.24355    AND    6.035075

COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE.
```

Figure 4. Computer generated output of results from *D. magna* results.

The methyl substituted compounds, 3-methylbenzothiophene and 5-methylbenzothiophene, both prompted a toxic response from the *Daphnia*. 3-Methylbenzothiophene was the more toxic of the two, having an average LC_{50} value of 2.4 mg/L whereas the average value for 5-methylbenzothiophene was 18.8 mg/L. The sulfone derivatives of these two compounds were not toxic to the *Daphnia* in any of the tests.

Of the three diones tested, only 7-methylbenzothiophene-2,3-dione was toxic, having an average LC_{50} value of 5.4 mg/L. When compared to the other compounds tested, benzothiophene-2,3-dione was the least toxic of the chemicals. There was no toxicity observed for 5-methylbenzothiophene-2,3-dione. A LC_{50} value for phenol has been reported as 9.6 mg/L with *Daphnia* (9). This is near the LC_{50} value of 8.3 mg/L found for phenol and falls within the 95% C.I. given in Table 7.

On a molar basis (Table 8) there are four compounds that are more toxic than phenol. These include benzothiophene sulfone, dibenzothiophene sulfoxide, 3-methylbenzothiophene and 7-methylbenzothiophene-2,3-dione. The remaining compounds either had LC_{50} values that were greater than phenol or were considered to be non-toxic.

There were two instances where the oxidized compounds were more toxic than their parent compounds, while in other cases the converse was true. For example, benzothiophene sulfone was approximately 9-times more toxic than benzothiophene.

Dibenzothiophene did not show any toxicity to the daphnids. This may be due to the low solubility of this OSC in aqueous solution. Eastmond *et al.* (26) observed that higher molecular weight aromatics (anthracene, benzo[a]anthracene, and chrysene) are non-toxic to *D. magna* and attributed these findings to their low solubilities. Of the two

dibenzothiophene derivatives tested, only dibenzothiophene sulfoxide showed toxicity with an average LC_{50} of 8.3 mg/L. The sulfone of dibenzothiophene did not show any toxicity and may be due to its low solubility (0.03 mM) in the reconstituted water used in the *Daphnia* test. The sulfone derivative of 3-methylbenzothiophene also did not show any toxicity in the *D. magna* test while the parent compounds had an average LC_{50} values of 16 and 18.8 mg/L respectively.

It is possible that the methylated benzothiophenes are prompting a toxic response in these invertebrates and the oxidized products are able to be excreted from the organisms without causing any damage to the invertebrate. This does not provide an explanation for the fact that the sulfone derivative of benzothiophene is much more toxic than the parent compound. Interestingly, benzothiophene sulfone was found to be more toxic to germinating cucumber seeds than benzothiophene (84). Indeed, at 100 mg/L, this sulfone was as phytotoxic as 2,4-dichlorophenoxyacetic acid (2,4-D).

Of the 2,3-dione derivatives, only 5-methylbenzothiophene-2,3-dione was determined to be non-toxic. Benzothiophene-2,3-dione was the least toxic of any of the chemicals showing a response with an average LC_{50} of 143 mg/L. The 2,3-dione derivative of 7-methylbenzothiophene was the most toxic of the diones with an average LC_{50} of 5.4 mg/L.

The reference toxicant phenol was used in this study to provide a comparison between the *Daphnia* and Microtox tests. In the *Daphnia* test, of the four compounds that were more toxic than phenol, three of them (benzothiophene sulfone, dibenzothiophene sulfoxide and 7-methylbenzothiophene-2,3-dione) were oxidized derivatives of the parent compounds.

Unfortunately there were some fairly large variations in the LC_{50} values for compounds, including dibenzothiophene sulfoxide, 3-methylbenzothiophene and 7-methylbenzothiophene-2,3-dione. Although these difference are large, some discrepancies are expected when dealing with invertebrates. A general health problem within the population may lead to an overestimate of LC_{50}. Conversely, test organisms that may have had a food source carried over into the test vessel may have had a chance to feed during the test and thus give an underestimation of the LC_{50}.

Eastmond *et al.* (26) found the LC_{50} values of benzothiophene and dibenzothiophene to be 63.7 and 0.466 mg/L respectively. The average value reported in this study for benzothiophene was 51.3 mg/L which is reasonably close to the value found by Eastmond *et al.* (26). Dibenzothiophene did not elicit a toxic response to *D. magna* in this study whereas Eastmond *et al.* (26) reported an LC_{50} value. This may have been due to differences in the respective *Daphnia* populations. The low solubility of dibenzothiophene may also account for the lack of a toxic response.

3.6 Microtox Testing of Supernatants of Cultures Grown in the Presence of Condensed Thiophenes (Isolate F)

The toxicity tests described in section 3.4 were limited to those compounds that can be purchased or chemically synthesized in high purity and sufficient quantity to use in the Microtox assay. However, many of the bacterial metabolites of benzothiophenes and dibenzothiophenes are very difficult to synthesize. Among these are the sulfoxides of benzothiophene and the methylbenzothiophenes (32, 60, 82) and 3-hydroxy-2-formylbenzothiophene (HFBT) which is a common metabolite of dibenzothiophene (59, 62, 71).

Because many of the condensed thiophenes are cometabolized by bacteria, the strategy used for these experiments was to grow bacterial cultures on a carbon source with one of the condensed thiophenes in the liquid medium as a co-metabolite. Then at various times, supernatant samples were removed and assayed using the Microtox method to detect changes in supernatant IC_{50} values caused by any undetected and unidentified metabolites that might be present in the batch culture supernatant.

3.6.1 Microtox Testing of Supernatants of Cultures Grown in the Presence of Benzothiophene

Figure 5 summarizes the toxicity data collected over a 144 h incubation period with *Pseudomonas* strain F grown on glutamic acid with 9.8 mg/L benzothiophene in the medium. The results are expressed as the percent by volume of the culture supernatant that gave a 50% reduction in the light emission from a suspension of *P. phosphoreum*. Initially, the supernatant of the active culture had an IC_{50} of 16% (v/v). This was in good agreement with

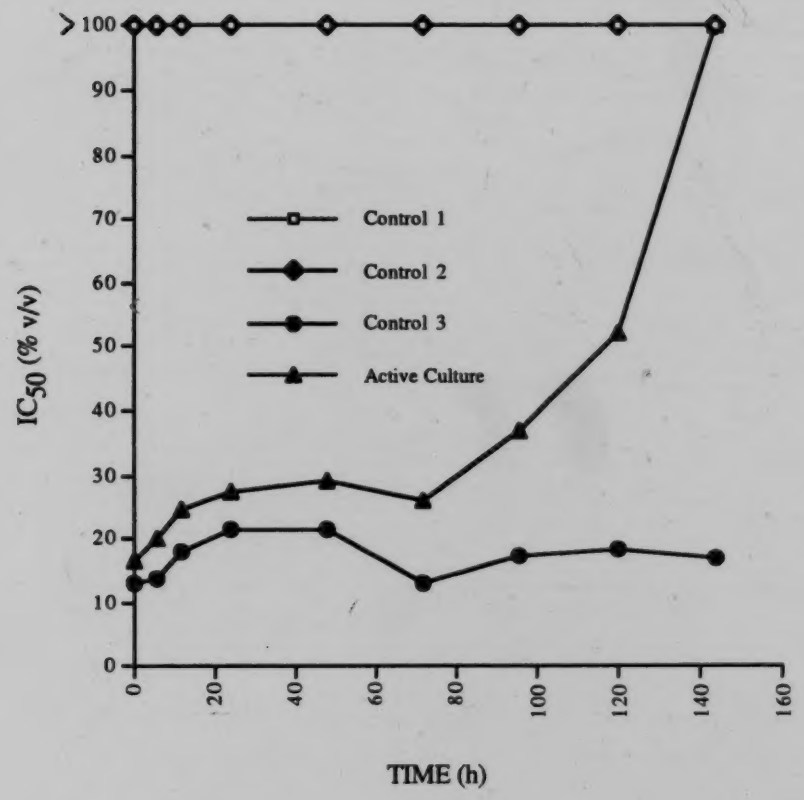

Figure 5. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 9.8 mg/L benzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, sterile control containing glutamic acid and benzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

the sterile control that contained benzothiophene (Control 3), which gave an IC_{50} value of 13% (v/v). No toxicity was observed for the other two controls (Controls 1 and 2), without benzothiophene, and the IC_{50} values were >100% (v/v). The IC_{50} values of these two controls remained at this level throughout the experiment. One of these (Control 2) was inoculated with strain F, which grew on the glutamic acid with no benzothiophene, the other was sterile with glutamic acid and benzothiophene. Because there was no difference between the measured toxicities of these two controls, the growth of strain F did not produce any detectable toxic compounds. On the following figures the endpoint is shown as a toxicity of 100%. The Microtox test gives a value of "Greater than 100%" when samples are determined to be non-toxic. Therefore, when a value of 100% is shown on the graph, it indicates a value of greater than 100%.

There was no obvious change in the toxicity of the benzothiophene-containing sterile control (Control 3) over the course of the experiment (Fig. 5). However, after 70 h of incubation, there was a marked decrease in the toxicity of the supernatant from the active culture that contained benzothiophene. By the time of the last sampling, the IC_{50} value was >100% (v/v), the same as the controls without benzothiophene. These results indicate that the cometabolism of benzothiophene was reducing the toxicity of this compound, and there was no evidence with the Microtox test that the metabolites being produced were increasing the toxicity of the supernatant.

Figure 6 summarizes the toxicity data collected over a 75 h incubation period. This experiment was a repeat of the experiment in Figure 5. In the second trial with benzothiophene (Fig. 6) the endpoint was reached at 60 h as opposed to 144 h in the first experiment (Fig. 5). Neither control 1 and 2 showed any indications of toxicity at any time during incubation.

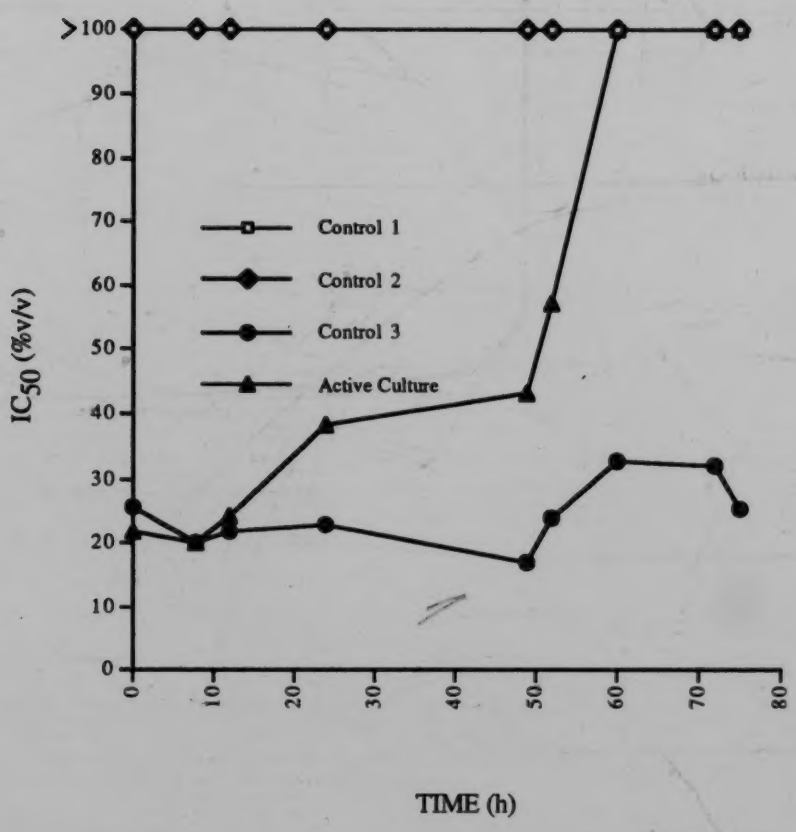

Figure 6. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 9.8 mg/L benzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, sterile control containing glutamic acid and benzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

At 8 h, both the active culture and Control 3 had approximately the same toxicity at 23% (v/v). By 24 h, the active culture had a toxicity of 38% while the control containing sterile medium and benzothiophene retained a toxicity of 23% (v/v). This control (Control 3) had a relatively constant toxicity throughout the experiment between 25 and 35%. By 60 h there was no observable toxicity remaining in the active culture.

3.6.2 Microtox Testing of Supernatants of Cultures Grown in the Presence of Dibenzothiophene

Figure 7 summarizes the toxicity data collected over a 48 h incubation time. Initially, the supernatant of the active culture had an IC_{50} of 67% (v/v). The sterile control that contained dibenzothiophene (Control 3), gave an initial IC_{50} value of 43% (v/v). However, there was virtually no difference between the IC_{50} values of the active culture and Control 3 at the 6 h and 12 h sampling times. Over the course of the experiment, no toxicity was observed for the other two controls (Controls 1 and 2), without dibenzothiophene, and the IC_{50} values were >100% (v/v).

The toxicity of the dibenzothiophene-containing sterile control (Control 3) remained fairly constant over the 48 h test period. However, after 12 h of incubation, there was a sharp decrease in the toxicity of the supernatant from the active culture that contained dibenzothiophene. Within 24 h of incubation, the IC_{50} value was >100% (v/v), the same as the controls without dibenzothiophene. Thus there was no indication that the co-metabolism of dibenzothiophene was producing metabolites that would increase the toxicity of the supernatant.

Figure 8 summarizes a repeat of the experiment presented in Fig. 3. There was no toxicity observed in Controls 1 and 2 throughout the course of

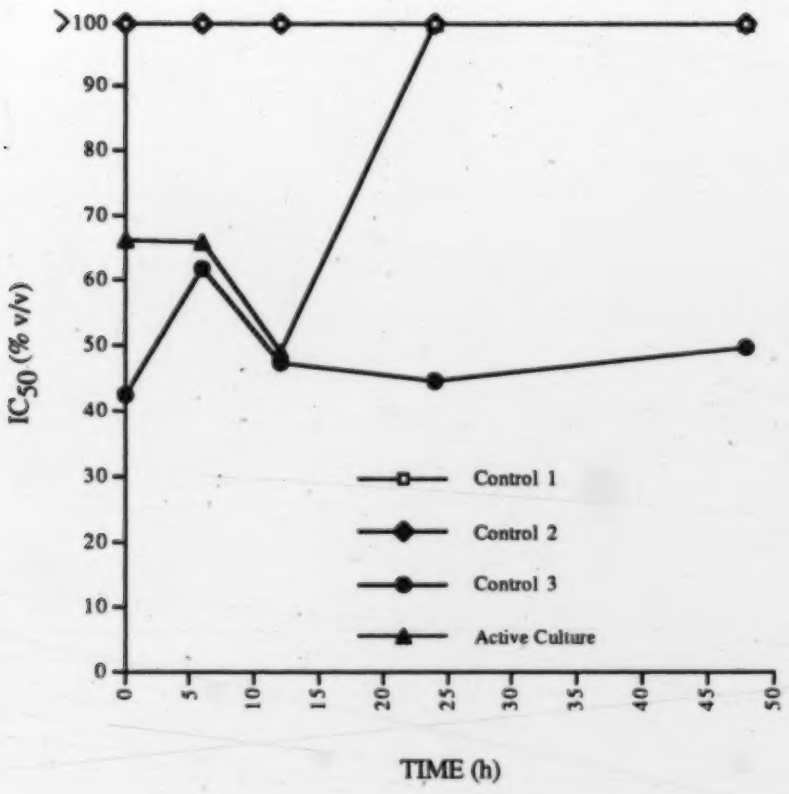

Figure 7. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 1.2 mg/L dibenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

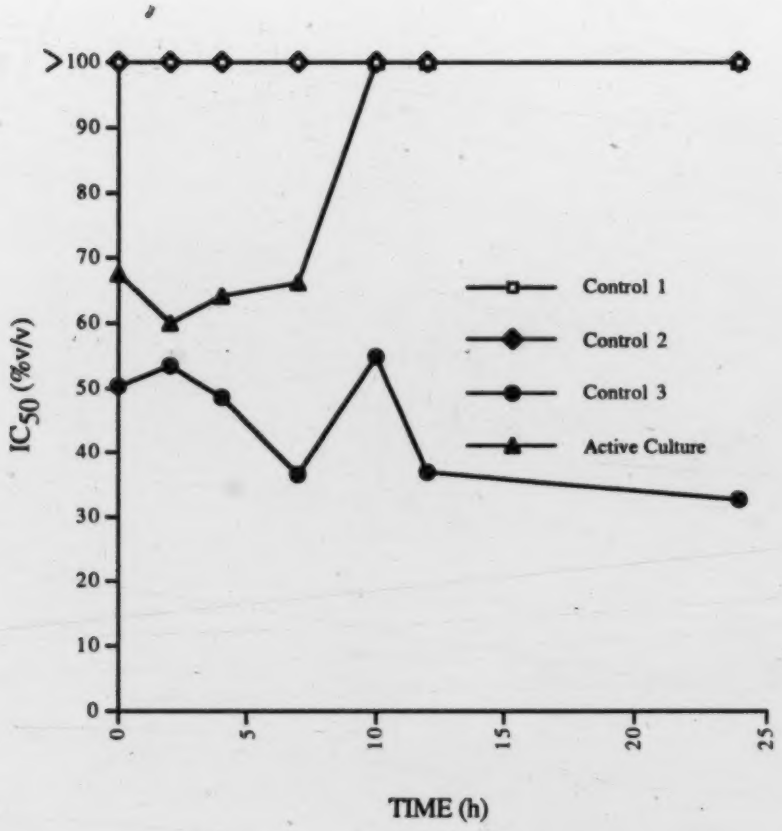

Figure 8. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 1.2 mg/L dibenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

the experiment. The active culture initially had a toxicity of 68% while the control containing dibenzothiophene (Control 3) had a toxicity value of 50% at time 0. After 10 h there was no toxicity observed in the active culture and the endpoint, 24 h, there was still no toxicity recorded.

3.6.3 Microtox Testing of Supernatants of Cultures Grown in the Presence of 3-Methylbenzothiophene

The results for this toxicity experiment are presented in Fig. 9. Only 27 h were required for completion of the test. For the first 4 h, the IC_{50} from the supernatant of the active culture was similar to that of the control containing 3-methylbenzothiophene. After 12 h of incubation, the active culture showed signs of toxicity reduction. The control flask with 3-methylbenzothiophene had a toxicity of 26% while the active culture had a toxicity of 43%.

After 24 h of incubation, there were no signs of toxicity in the supernatant from the active culture. As with the other compounds tested, there were no signs of toxicity in the first two control flasks.

The results from a repeat of this experiment are presented in Fig. 10. Fifty one hours were required for completion of this test. For the first 10 h the active culture and Control 3 showed similar toxicity values. After 22 h the active culture started to show signs of toxicity reduction as compared to the control containing sterile 3-methylbenzothiophene. As with the other toxicity experiments, there was no observable toxicity in Controls 1 and 2.

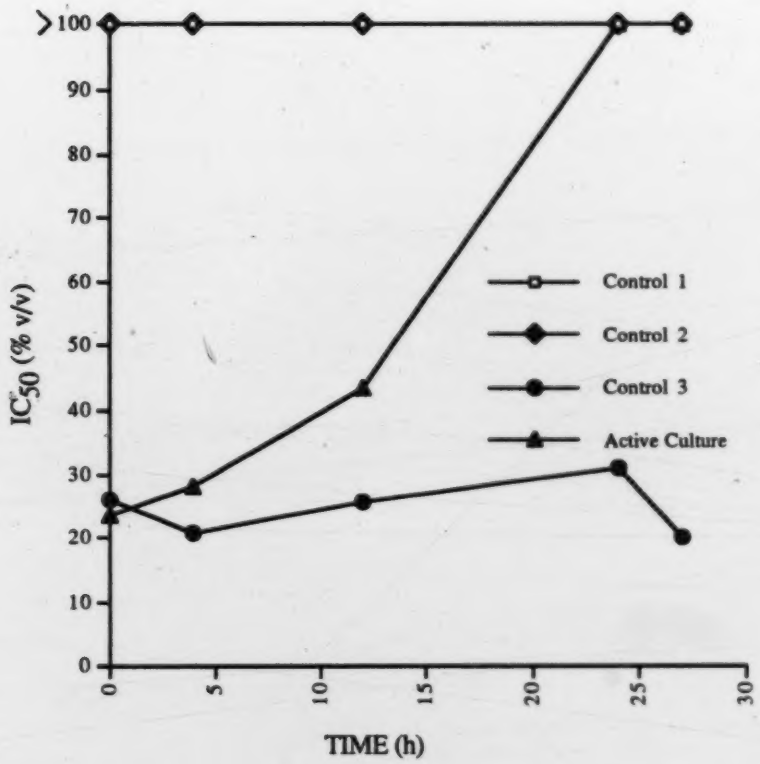

Figure 9. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 4 mg/L 3-methylbenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

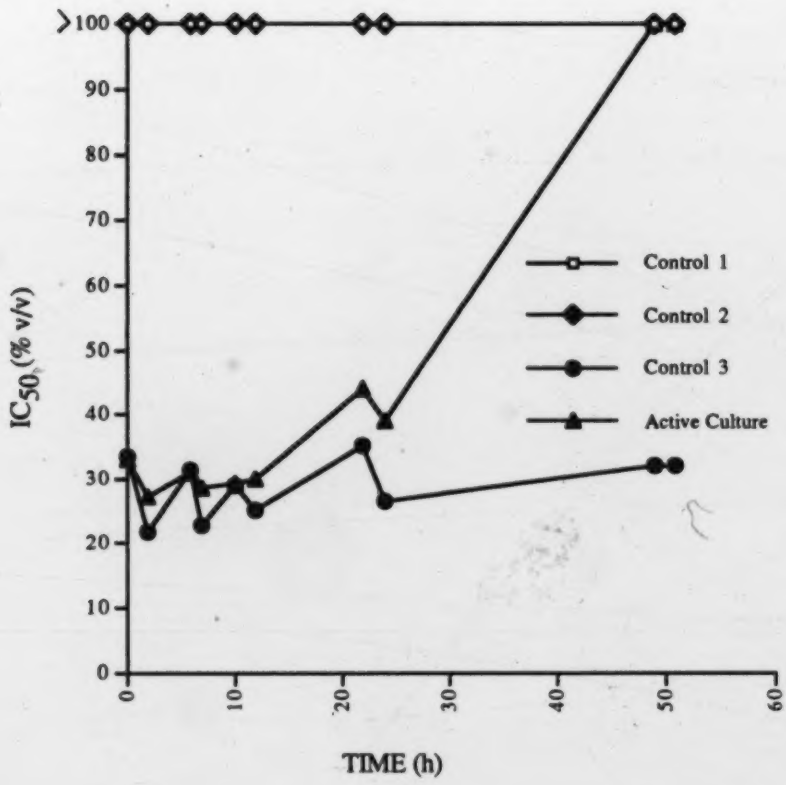

Figure 10. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 4 mg/L 3-methylbenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

3.6.4 Microtox Testing of Supernatants of Cultures Grown in the Presence of 5-Methylbenzothiophene

As with benzothiophene and 3-methylbenzothiophene, a saturated solution of 5-methylbenzothiophene was prepared in sterile B+NP medium containing glutamic acid. 5-Methylbenzothiophene was added to give a final concentration of 4.5 mg/L. Figure 11 summarizes the toxicity data collected over a 101 h incubation period. The reduction in toxicity followed the same trend as that of the previous three compounds. Until 24 h, the toxicity of the supernatant from the active culture was close to that of the control flask containing 5-methylbenzothiophene but without any inoculum. After this time, the active culture started to show signs of detoxification until there was no longer any toxicity observed after 70 h.

The results of a repeat of this test are presented in Fig. 12. One hundred and twelve hours were required for this test to go to completion. The control containing sterile 5-methylbenzothiophene without any inoculum (Control 3) and the active culture had similar toxicity values for the first 20 h. After this the active culture started to show signs of detoxification and after 68 h there were no signs of toxicity in the culture supernatant.

3.7 Microtox Testing of Supernatants of Cultures Grown in the Presence of Condensed Thiophenes (Isolate W1)

For this part of the project, it was thought that the results would be somewhat similar to that of the work with isolate F. However isolate W1 was grown in B+NP medium with glutamic acid, the medium turned a lime green colour after approximately one day.

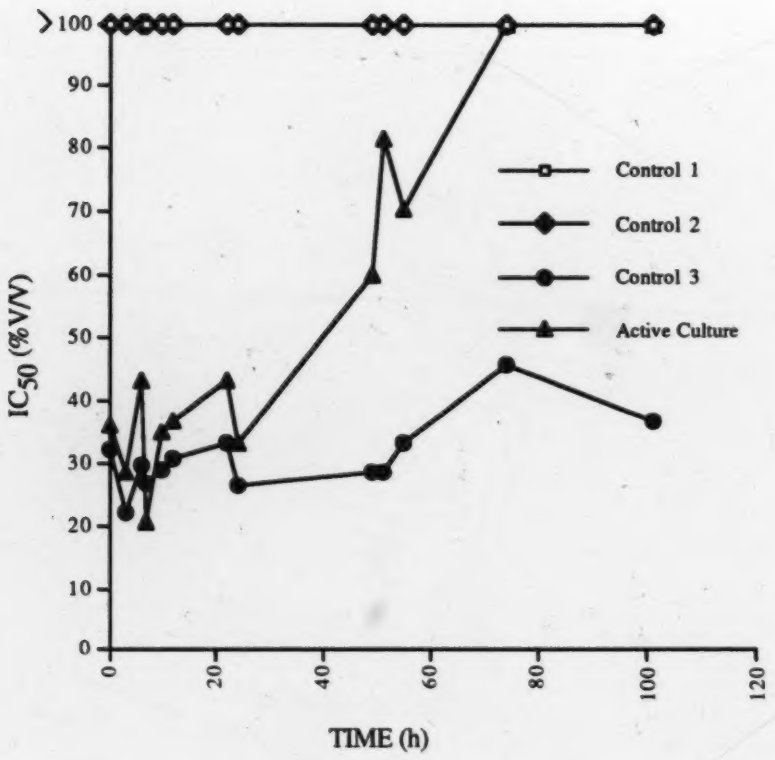

Figure 11. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 4.5 mg/L 5-methylbenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

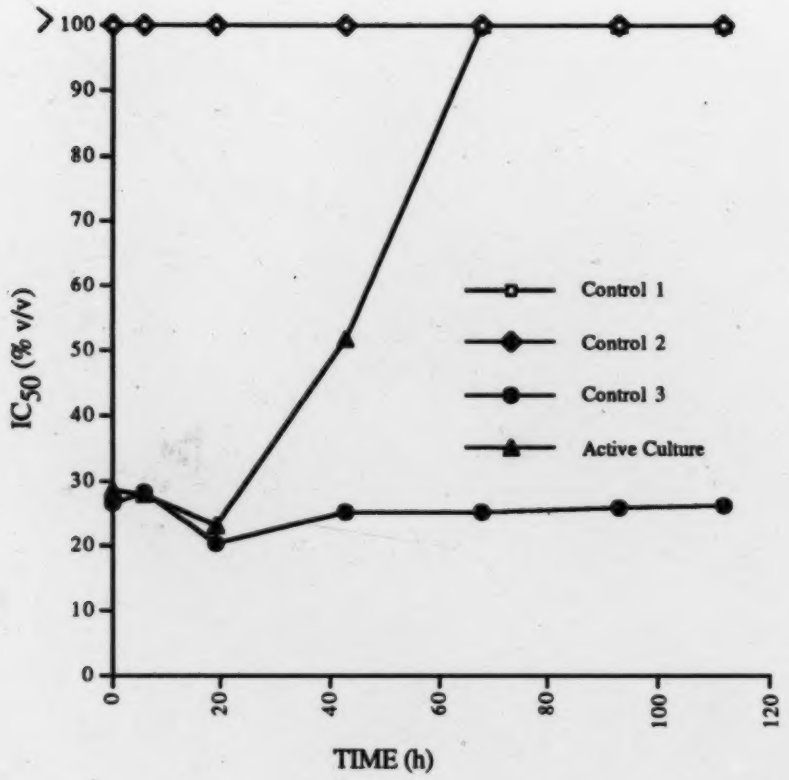

Figure 12. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain F grown on glutamic acid in the presence of 4.5 mg/L 5-methylbenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

A UV scan of this culture showed a peak absorbance approximately 400 to 500 nm. Unfortunately, the Microtox Model 500 takes its readings at 490 nm. Therefore in the initial stages of these experiments the control which only had isolate F growing on glutamic acid, showed what appeared to be a toxic response. The reason for this may have been attributed to the fact that the green color was masking the light output from each of the test flasks. A notable point is that after 2 weeks, the color of the culture changed from the lime green to a pale yellow. Accompanying this change in color was the reduction in the apparent toxicity. Unfortunately it is not known whether the apparent reduction in toxicity was due to the change in color or from the action of isolate W1 on the condensed thiophene being tested.

This then led to the subsequent termination of this experiment. With the color correction procedure utilized by the Microtox system this problem could be overcome. Due to the lack of equipment for this procedure, the color correction was not pursued.

Figure 13 summarizes the data from isolate W1 grown in the presence of dibenzothiophene. Only one figure is included from the tests with isolate W1, but this figure is representative of the other tests with the three remaining condensed thiophenes.

3.8 Toxicity Testing of Supernatants from Cultures Grown in the Presence of OSC

There was one common feature of all the tests dealing with the toxicity of the supernatants of active cultures, a plateau of toxicity. This plateau, an area where the toxicity of the active culture growing in the presence of an OSC closely resembled the toxicity of a control that contained the OSC but without the addition of any inoculum, remained for anywhere from 3 to 24 h.

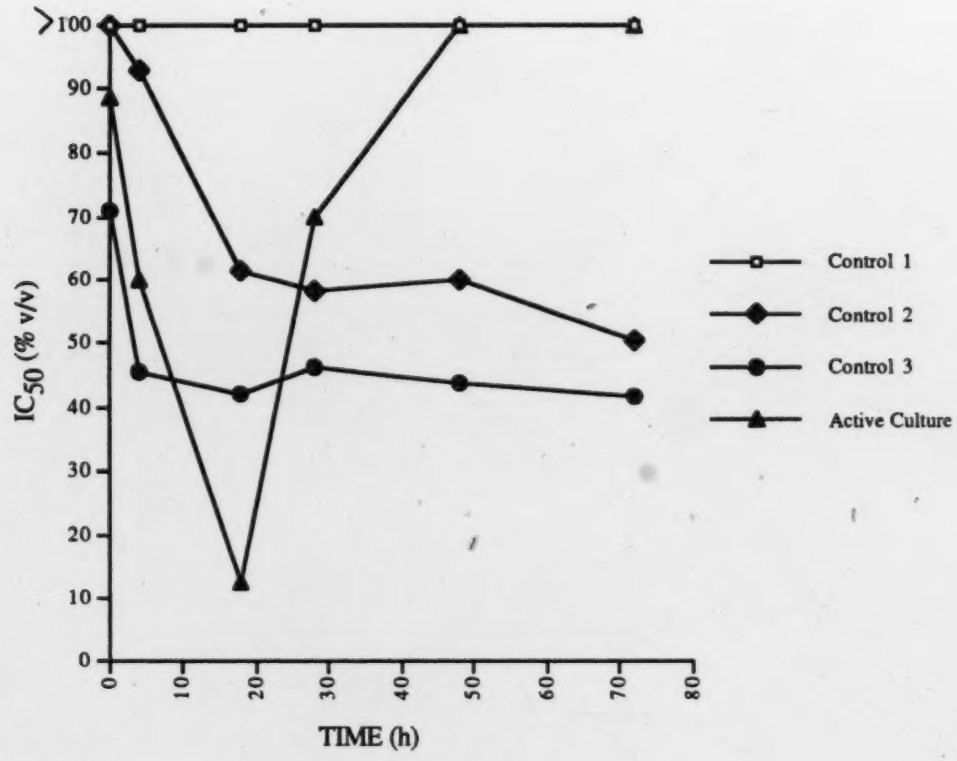

Figure 13. Microtox testing of supernatant samples from a culture of *Pseudomonas* strain W1 grown on glutamic acid in the presence of 1.2 mg/L dibenzothiophene (Active Culture). Control 1, a sterile control containing glutamic acid only; Control 2, a live culture grown on glutamic acid; Control 3, a sterile control containing glutamic acid and dibenzothiophene. Where 100% (v/v) toxicity was reached, it is actually greater than 100% as determined by the Microtox test.

These plateaus may be due to an excess of compound present and the inoculum not being able to cope with its presence. More likely these plateaus are the result of the organisms' lag time once they were introduced into the active culture flask. Initially the organisms would be present in relatively low numbers and detoxification would be at low levels. Once the population was actively growing and there were large numbers present, would the detoxification of the OSC become readily noticeable.

4 Discussion

4.1 Aqueous solubilities of OSC and Polycyclic Aromatic Hydrocarbons (PAH)

Statistical analyses of the results in Table 4 showed that centrifugation of the samples gave lower concentrations of the test compounds, presumably due to adsorption of the compounds to the centrifuge tubes. Duncan's multiple range test ($P<0.05$) showed that centrifugation in the plastic tubes gave lower concentrations in 9 of the 10 test cases, whereas centrifugation in the glass tubes gave lower concentrations in 8 of the 10 test cases. Clearly, the solubility concentrations must be determined on samples from the flask in which the saturated solution was prepared.

From Table 4 it is clear that the oxidized derivatives of the OSC are more soluble than the parent compounds. The same trend follows for the methylated benzothiophenes used in this experiment. These results seem to be consistent with similar compounds. The PAH also have low solubilities and it would be expected that the OSC examined in this study would show similar results. The addition of a sulfur atom to similar compounds would not likely change the non-sulfolated and sulfolated compounds' solubility. Evidence of this is that the solubility of fluorene was reported to be 1.98 mg/L (65) as compared to an aqueous solubility of 1.7 mg/L for dibenzothiophene (94). A reported value for indan is 110 mg/L (66) which is quite similar to the reported value of 113 mg/L for benzothiophene (94). Table 9 summarizes the aqueous solubilities.

These solubility results show that at even very low solubilities, there are strong toxic effects. Therefore it is important that even small quantities of these compounds do not find their way into the environment. Due to their low solubility, these compounds could persist for prolonged periods of time and

Table 9. Aqueous solubilities of some condensed thiophenes and their oxidation products.

	Aqueous Solubility					
	(mg/L)			(mM)		
	Mean	SD	n	Mean	SD	n
Benzothiophene	159	4.2	2	1.2	0.03	2
Benzothiophene sulfone	544	41	3	3.3	0.24	3
3-Methylbenzothiophene	49	0.2	2	0.33	0.001	2
3-Methylbenzothiophene sulfone	388	39	3	2.1	0.21	3
5-Methylbenzothiophene	46.2	7.8	2	0.31	0.05	2
5-Methylbenzothiophene sulfone	399	9.1	2	2.2	0.21	2
Dibenzothiophene	1.05	0.2	2	0.006	0.001	2
Dibenzothiophene sulfoxide	325	1.4	2	1.6	0.007	2
Dibenzothiophene sulfone	5.8	0.03	2	0.03	0.003	2

slowly be released into the aqueous phase.

It is expected that the oxidized metabolites would be more soluble than the parent compounds because they would become more polar and therefore increasing solubility. From these results, it would be expected that other methylated derivatives of benzothiophene and dibenzothiophene would be more soluble than their parent compounds.

4.2 Comparison of toxicities of parent condensed thiophenes and their oxidized derivatives

Based on Microtox analyses of the individual sulfur compounds, there was a general trend observed. The oxidized derivatives of the parent compounds were less toxic than the parent compounds. From the results from DMSO solutions, all of the oxidized compounds with the exception of 3-methylbenzothiophene sulfone, were less toxic than their parent compounds (Table 3). Thus the methyl group may be a cause of the toxic response when the parent is oxidized. It may be possible that other alkyl-isomers of benzothiophene and dibenzothiophene would have oxidized compounds that are more toxic than their parent compounds.

Table 10 summarizes the results from aqueous solutions of the OSC. From this table it is possible to see that the all of the oxidized derivatives are less toxic than the parent compounds. The aqueous solutions provided the expected results of the oxidized compounds being less toxic than their respective parent compounds. When non-sulfolated compounds are examined, the same trend is followed. A reported IC_{50} value for naphthalene is 0.93 mg/L (55). Some of its known metabolites, naphthol, catechol, and salicylic acid have reported IC_{50} values of 3.7 mg/L, 29.6 mg/L,

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Table 10. Toxicities of some condensed thiophenes and some of their oxidation products. IC_{50} values are based on 15-min Microtox assay of aqueous solutions

	IC_{50}					
	(mg/L)			(μM)		
	Mean	SD	n	Mean	SD	n
Benzothiophene	1.7	0.47	2	10	4	2
Benzothiophene sulfone	7.0	2.81	3	40	17	3
Benzothiophene-2,3-dione[a]	184	129	2	690	480	2
3-Methylbenzothiophene	0.67	0.24	2	4.5	2	2
3-Methylbenzothiophene sulfone	31.6	6.82	3	175	38	3
5-Methylbenzothiophene	0.70	0.16	2	5	1	2
5-Methylbenzothiophene sulfone	33.7	9.59	2	187	53	2
5-Methylbenzothiophene-2,3-dione[a]	116	11.2	2	417	41	2
7-Methylbenzothiophene-2,3-dione[a]	75.3	25.5	2	272	92	2
Dibenzothiophene	0.16	0.025	2	0.9	0.1	2
Dibenzothiophene sulfoxide	2.10	1.85	2	11	9	2
Dibenzothiophene sulfone	>5.8[b]	0.0	2	>27[b]	0.0	2

[a]At the neutral pH of the toxicity test, the 2,3-diones exist as very soluble 2-mercaptophenylglyoxalates (27). Thus, these compounds were prepared to the concentrations stated.

[b]No toxicity observed, thus the IC_{50} is greater than the maximum concentration tested and given.

and 214 mg/L respectively (55). This illustrates that the oxidation of the PAH naphthalene, yields metabolites that are less toxic than the parent compound, as was observed with the OSC studied in this project.

4.3 Comparison of Parent Compounds to their Oxidized Derivatives in the *D. magna* Toxicity Test

For the *D. magna* tests, all of the stock solutions of the OSC, except those of the 2,3-diones, were prepared as saturated aqueous solutions. After the completion of the *Daphnia* assays, the stock solutions were stored for various times (typically a few weeks) prior to analysis by HPLC to determine the concentrations of the OSC in solution. The results from the replicate stock solutions were often quite variable. For example, the concentrations of benzothiophene were measured to be 158 and 127 mg/L (Table 7). The former value agreed closely with the aqueous solubility of benzothiophene reported to be 159 mg/L (Table 9), but the later was much lower than expected for a saturated solution. Thus, the integrity of the stored stock solution was questioned.

Thus, it was decided to recalculate the LC_{50} values from the *D. magna* tests, and these are summarized in Table 11. For each compound, the LC_{50} values were calculated by multiplying its aqueous solubility, given in Table 9, by the LC_{50}, expressed as (%v/v) in Table 7. From these calculations, the mean LC_{50} value for each compound is given in Table 11, expressed in mg/L and μM.

Based on molar concentrations benzothiophene sulfone was approximately 5-times more toxic than benzothiophene. Dibenzothiophene did not show any toxicity to the daphnids. This may be due to the low

solubility of this OSC in aqueous solution. The only dibenzothiophene derivative to show any toxicity was dibenzothiophene sulfoxide which had

Table 11. Toxicities of some condensed thiophenes and some of their oxidation products. LC_{50} values are based on 48-h *Daphnia magna* assay of aqueous solutions

	LC_{50} (mg/L)			LC_{50} (μM)		
	Mean	SD	n	Mean	SD	n
Benzothiophene	59	15.9	2	440	120	2
Benzothiophene sulfone	14	3.6	2	86	20	2
Benzothiophene-2,3-dione[a]	270	90	2	1000	340	2
3-Methylbenzothiophene	2.6	2.09	2	18	10	2
3-Methylbenzothiophene sulfone	NT[b]					
5-Methylbenzothiophene	15	3.89	2	99	30	2
5-Methylbenzothiophene sulfone	NT[b]					
5-Methylbenzothiophene-2,3-dione[a]	NT[b]					
7-Methylbenzothiophene-2,3-dione[a]	13	9.16	2	46	30	2
Dibenzothiophene	NT[b]					
Dibenzothiophene sulfoxide	22	12.37	2	110	60	2
Dibenzothiophene sulfone	NT[b]					

[a] At the neutral pH of the toxicity test, the 2,3-diones exist as very soluble 2-mercaptophenyl-glyoxalates (27). Thus, these compounds were prepared to the concentrations stated.

[b] No toxicity observed, thus the IC_{50} is greater than the maximum concentration tested and given.

an average LC_{50} of 22 mg/L (Table 11). The sulfone of dibenzothiophene did not show any toxicity and may be due to its low solubility in the reconstituted water used in the *Daphnia* test.

The sulfone derivative of 3-methylbenzothiophene also did not show any toxicity to the *D. magna* test. Similarly, there was no toxicity observed for 5-methylbenzothiophene sulfone while the parent compound had an average toxicity of 15 mg/L (Table 11). It is possible that the methylated benzothiophenes are prompting a toxic response in these invertebrates and the oxidized products are able to be excreted from the organisms without causing any damage to the invertebrate. This does not provide an explanation for the fact that the sulfone derivative of benzothiophene is

Of the 2,3-dione derivatives, only 5-methylbenzothiophene-2,3-dione was determined to be non-toxic. Benzothiophene-2,3-dione was the least toxic of any of the chemicals tested with an average LC_{50} of 143 mg/L. The 2,3-dione derivative of 7-methylbenzothiophene was the most toxic of the diones with an average LC_{50} of 5.4 mg/L.

These variations do not lend themselves to providing a clear statement as to the comparison of toxicity between the parent and derivative compounds. It would be expected that the derivatives of the compounds would be less toxic in all instances. The oxidized nature of these compounds should make them easier to excrete from the organisms.

4.4 Comparison of IC_{50} and LC_{50} Values from the Microtox and *Daphnia* Tests Based on Molar Concentrations

Table 10 summarizes the molar concentrations of the IC_{50} values from the Microtox test, while the molar concentrations of the LC_{50} values from the *Daphnia* tests are presented in Table 11. In the Microtox test

benzothiophene had an average IC_{50} value of 10 μM while in the *Daphnia* test the LC_{50} value was 440 μM. This is a substantial difference between the two tests as benzothiophene is 44-times less toxic to the invertebrate than to the microbial system. The oxidized derivative of benzothiophene, the sulfone, did not have a such a wide difference in toxicities between the two tests. In the Microtox test the IC_{50} was 40 μM while the LC_{50} value was 86 μM. It is important to note that benzothiophene sulfone was one of the more toxic compounds to the *Daphnia* while in the Microtox test it was one of the less toxic compounds.

Although dibenzothiophene was one of the most toxic compounds testing in the Microtox test, there was no toxicity observed in the *Daphnia* test. As mentioned previously, this may be due to the low solubility of the compound in aqueous solution. In both toxicity tests, there was no toxicity observed for dibenzothiophene sulfone. The sulfoxide of dibenzothiophene had an IC_{50} value of 11 μM while in the *Daphnia* test this compound had an LC_{50} of 110 μM. Thus this compound is 10-times less toxic to the *Daphnia* as opposed to the microorganism used in the Microtox test.

The IC_{50} value for 3-methylbenzothiophene was 45 μM in the Microtox test while in the *Daphnia* test this compound had an LC_{50} value of 18 μM. Again the higher trophic level of test provided a lower value of toxicity. In the Microtox test, the sulfone derivative of 3-methylbenzothiophene had one of the higher IC_{50} values of 175 μM. There was no observable toxicity in the *Daphnia* test. It can be suggested that this compound is one of the lesser risks to the environment.

Again with 5-methylbenzothiophene the same pattern occurs. There is substantial difference between the two tests. An IC_{50} value of 5 μM was observed in the Microtox test while in the *Daphnia* test the LC_{50} value was

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Again with 5-methylbenzothiophene the same pattern occurs. There is substantial difference between the two tests. An IC_{50} value of 5 μM was observed in the Microtox test while in the *Daphnia* test the LC_{50} value was

99 μM. This compound was 20-times less toxic to the *Daphnia* than to the microorganism in the Microtox test. The sulfone derivative of this compound was not toxic to the *Daphnia* and was one of the least toxic in the Microtox test.

In the Microtox test all of the diones are generally not as toxic as other compounds. These compounds had very high IC_{50} values (benzothiophene-2,3-dione, 690 μM; 5-methylbenzothiophene-2,3-dione, 417 μM; 7-methylbenzothiophene-2,3-dione, 272 μM) thus indicating low toxicity. In the *Daphnia* test 7-methylbenzothiophene-2,3-dione was relatively toxic with an LC_{50} value of 46 μM, as compared to the other compounds. Benzothiophene-2,3-dione had the highest LC_{50} value of all compounds tested in the *Daphnia* test at 1000 μM. In the Microtox test this compound had an IC_{50} value of 690 μM. In both tests this compound was not as toxic as some of the other compounds tested. In the Microtox test 5-methylbenzothiophene-2,3-dione had an IC_{50} value of 417 μM while in the *Daphnia* test there was no toxicity observed.

It is clear that, with the exception of benzothiophene sulfone, all of the compounds tested were less toxic to the invertebrate, *D. magna*, than they were to the microorganism *P. phosphoreum*. As the determination of toxicity in the Microtox test is the reduction in light output, any compound which interfere with luciferase in the *P. phosphoreum*, may produce results that overestimate the true toxicity of the compound of interest. Most likely it is the compounds interacting with the cellular structure that is causing the toxic response. The compounds may interfere with the electron transport chain which would lead to cell death. Alternatively, the lipophilic nature of these compounds could cause them to react with the cell membrane and structurally damage the cell, resulting in death.

The smaller size and larger surface area of the microorganisms used in the Microtox test may make them more susceptible to the compounds at the low solubilities observed for these compounds. In an acute test, the bacteria are immediately challenged with a solution containing the OSC and they may have difficulty dealing with this sudden exposure thus leading to a reduction in light output. In the *Daphnia* test the organisms may be able to deal with the immediate exposure to the low concentrations of OSC in solution as they are substantially larger. It would be interesting to see if there is any toxicity observed in a chronic test with *Daphnia* to the compounds that did not show any toxicity in the 48-h static test.

It is possible that the cytochrome P-450 enzyme system found in eukaryotes is better able to convert toxic compounds into less toxic derivatives. If this were the case then it would explain the general trend for the compounds to be less toxic to the *Daphnia* as opposed to the microorganism in the Microtox test.

4.5 Toxicity Testing of Supernatants from Cultures Growing in the Presence of OSC

In the two tests with isolate F growing in the presence of benzothiophene the process of detoxification followed a similar pattern. In the first test the endpoint was reached after 144 h while in the second test the endpoint was reached in only 75 h. The shorter time required to reach the endpoint in the second test may be due to a population that was under better conditions to detoxify benzothiophene. As these tests were conducted at room temperature, there would be fluctuations in the ambient temperature. These temperature fluctuations may be small but it is possible that they may influence the rate of detoxification. In order to correct for this, two sets of

experiments could be run concurrently. One set of flasks at room temperature and another set at controlled conditions.

In both instances, there was a period where the toxicity of the active culture was similar to the control containing benzothiophene without any inoculum (Control 3). This was followed by a period of rapid detoxification leading to the endpoint.

Tests with dibenzothiophene were similar to those of benzothiophene except that the time required to reach the endpoint was less. In the first test, the supernatant was considered non-toxic after 24 h while in the second test there was no sign of toxicity in the active culture flask after 10 h. This shorter time may be due to the fact that there was only 0.24 mg of dibenzothiophene present in the flask. This relatively small amount of compound may account for the short time required for the bacterial population to detoxify the compound even though it is quite toxic.

In the first test with 3-methylbenzothiophene there was a gradual decrease in toxicity in the active culture as compared to the control containing 3-methylbenzothiophene. The second test saw the active culture more closely follow the toxicity of the control containing 3-methylbenzothiophene for 24 h. Then there was a rapid decrease in toxicity until reaching the endpoint.

The longest amount of time of all the tests was required to reach the endpoint for tests with 5-methylbenzothiophene. Both tests required just over 60 h for the active culture flask to be non toxic. In both tests, the toxicity of the active culture flask closely resembled the toxicity of the control containing 5-methylbenzothiophene for the first 24 h and then there was steady decline in the toxicity of the active culture.

4.6 Implications for Bioremediation and Biodegradation

From the results presented here there are some important items to mention in regards to the degradation of toxic chemicals in the natural environment. All of the oxidation products of select benzothiophenes were less toxic than the parent compounds when tested with the Microtox system. This indicates that at a low trophic level, the biodegradation of these OSC will not result in the production of more harmful metabolites. This is a very important aspect for biodegradation purposes. If the by-products of microbial degradation were toxic, then as the initial compounds were degraded, toxic by-products would result in the demise of the bacterial population. This would be a great inhibitor to microbial degradation of the contaminated site.

If through microbial degradation more toxic by-products were produced, then bioremediation projects would in a sense be doing more harm than good. If by trying to clean up a contaminated site, the site actually became more toxic, then there would be little reason to pursue the process. If these metabolites were more toxic and were intermediate products, their subsequent biodegradation led to non-toxic end products, then there would be no problems associated with the bioremediation.

On a higher trophic level, invertebrates, the results were generally in line with the Microtox data. On the whole, the parent compounds were more toxic than the oxidized metabolites. Benzothiophene sulfone is the one exception where conclusive data were found. This OSC was more toxic than the parent compound to the invertebrate, *Daphnia magna*. There were no toxic results for dibenzothiophene, presumably due to the low solubility of the compound.

In this instance, dibenzothiophene would more likely be adsorbed to soil particles in soil systems rather than being in the aqueous phase or

dissolved in a co-contaminant such as petroleum. This is an important aspect to consider in the remediation of a contaminated site. While most of these compounds are soluble enough to be concerned about the water table, it is important to consider those compounds that will adhere to particles in the contaminated site.

As a general extrapolation, through analyses of some OSC the process of bioremediation appears to have a positive effect on the clean up of contaminated sites. Only a few of the many compounds found in petroleum products were tested in this study. Further analysis is necessary to generate a more thorough picture of the bioremediation process.

5 Summary

The data presented show that the oxidized derivatives of some benzothiophenes and dibenzothiophene are more soluble than the parent compounds. This follows the reasoning that by making a compound more polar, its water solubility is increased. Other metabolites of these compounds would be expected to be more soluble than the parent compound as long as they are being oxidized.

Microtox and *D. magna* analyses showed that oxidized metabolites studied are less toxic than their parent compounds. This is encouraging because the transformation of a contaminant to a more harmful product would be counter productive to bioremediation processes. Reduction in toxicity of these degradation compounds is very important in a polluted site. If the normal flora were to transform these compound into something more toxic, then there would be an increase in the contamination problem.

If there were more toxic metabolites produced, it seems likely that they would be further broken down in the normal metabolic pathway of the organisms present. Eventually, the compounds would be converted to carbon dioxide and water.

Using the *D. magna* toxicity test, it was possible to see that the Microtox was generally more sensitive to the thiophenes tested. There was still a noticeable toxic response in many of the thiophenes to the *D. magna* test. This indicates that these compounds can still illicit a toxic response in a higher trophic level.

By examining the supernatants cultures growing on select thiophenes, it was possible to see if there were any, as yet unidentified metabolites, that would produce a toxic response. The rapid reduction in

toxicity of supernatants with *Pseudomonas* isolate F, show that there were no metabolites producing a noticeable toxic effect.

6 References

1. Alexander, M. *Biodegradation and Bioremediation*, San Diego: Academic Press, 1994, p. 41-49.

2. Aprill, W., Sims, R.C., Sims, J.L. and Matthews, J.E. Assessing detoxification and degradation of wood preserving and petroleum wastes in contaminated soil. *Waste Mgmt. Res.* 8:45-65, 1990.

3. Arbuckle, W.B. and Alleman, J.E. Effluent toxicity testing using nitrifiers and Microtox. *Wat. Environ. Res.* 64:263-267, 1992.

4. Atlas, R.M. Effects of temperature and crude oil composition on petroleum degradation. *Appl. Microbiol.* 30:396-403, 1975.

5. Atlas, R.M. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Rev.* 45:180-209, 1981.

6. Atlas, R.M. and Bartha, R. Degradation and mineralization of petroleum in sea water: Limitation by nitrogen and phosphorus. *Biotechnol. Bioeng.* 14:309-318, 1972.

7. Atlas, R.M. and Bartha, R. Biodegradation of petroleum in seawater at low temperatures. *Can. J. Microbiol.* 18:1851-1855, 1972.

8. Atlas, R.M. and Bartha, R. Stimulated biodegradation of oil slicks using oleophilic fertilizers. *Environ. Sci. Technol.* 7:538-541, 1973.

9. Babich, H. and Davis, D.L. Phenol: A review of environmental and health risks. *Regul. Toxic. Pharmacol.* 1:90-109, 1981.

10. Baumann, P. and Baumann, L. Genus *Photobacterium* Beijerinck, 1889, 401^{AL}. In: *Bergey's Manual of Systematic Bacteriology, Vol. 1*, edited by Krieg, N.R. and Holt, J.G. Baltimore, MD: Williams and Wilkins, 1984, p. 539-545.

11. Berthou, F. and Vignier, V. Analysis and fate of dibenzothiophene derivatives in the marine environment. *Intern. J. Environ. Anal. Chem.* 27:81-96, 1986.

12. Bohonos, N., Chou, T.W. and Spanggord, R.J. Some observations on biodegradation of pollutants in aquatic systems. *Jpn. J. Antibiot.* 30 (Suppl.):275-285, 1977.

13. Bossert, I. and Bartha, R. The Fate of Petroleum in Soil Ecosystems. In: *Petroleum Microbiology*, edited by Atlas, R.M. New York: MacMillian Publishing Company, 1984, p. 435-474.

14. Brock, T.D. and Madigan, M.T. *Biology of Microorganisms*, Englewood Cliffs, New Jersey:Prentice Hall, 1991. 6th Edition.

15. Castrillón, J. The controlled oxidation of organic sulfides to sulfoxides by means of iodosobenzene diacetate. *Rev. Latinoamer. Quim.* 13:102-103, 1982.

16. Chen, C.I., Reinsel, M.A. and Mueller, R.F. Kinetic investigation of microbial souring in porous media using microbial consortia from oil reservoirs. *Biotechnol. Bioeng.* 44:263-269, 1994.

17. Chou, C.C. and Que Hee, S.S. Microtox EC_{50} values for drinking water by-products produced by ozonolysis. *Ecotox. Environ. Saf.* 23:355-363, 1992.

18. Colwell, R.R. and Walker, J.D. Ecological aspects of microbial degradation of petroleum in the marine environment. *CRC Crit. Rev. Microbiol.* 5:423-445, 1977.

19. Cooney, J.J. The fate of petroleum pollutants in freshwater ecosystems. In: *Petroleum Microbiology*, edited by Atlas, R.M. New York: MacMillian Publishing Company, 1984, p. 399-434.

20. Crawford, D.L. and Gupta, R.K. Oxidation of dibenzothiophene by *Cunninghamella elegans*. *Curr. Microbiol.* 21:229-231, 1990.

21. Dasappa, S.M. and Loehr, R.C. Toxicity reduction in contaminated soil bioremediation processes. *Water Res.* 25:1121-1130, 1991.

22. Davis, S.J. and Gibbs, C.F. The effect of weathering on a crude oil residue exposed at sea. *Water Res.* 9:275-285, 1975.

23. Demuth, S., Casillas, E., Wolfe, D.A. and McCain, B.B. Toxicity of saline and organic solvent extracts of sediments from Boston Harbour, Massachusetts and the Hudson River-Raritan Bay Estuary, New York using the Microtox bioassay. *Arch. Environ. Contam. Toxicol.* 25:377-386, 1993.

24. Dibble, J.T. and Bartha, R. Effects of environmental parameters on the biodegradation of oil sludge. *Appl. Environ. Microbiol.* 37:729-739, 1979.

25. Dillon, T.M., Neff, J.M. and Warner, J.S. Toxicity of sublethal effects of No. 2 fuel oil on the supralittoral isopod *Lygia exotica*. *Bull. Environ. Contam. Toxicol.* 20:320-327, 1978.

26. Eastmond, D.A., Booth, G.M. and Lee, M.L. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles in *Daphnia magna*. *Arch. Environ. Contam. Toxicol.* 13:105-111, 1984.

27. Eaton, R.W. and Nitterauer, J.D. Biotransformation of benzothiophene by isopropylbenzene-degrading bacteria. *J. Bacteriol.* 176:3992-4002, 1994.

28. Eisman, M.P, Landon-Arnold, S. and Swindoll, C.M. Determination of petroleum hydrocarbon toxicity with Microtox. *Bull. Environ. Contam. Toxicol.* 47:811-816, 1991.

29. Environment Canada, *Biological Test Method: Toxicity Test Using Luminescent Bacteria (Photobacterium phosphoreum)*, Ottawa:Environment Canada, 1992.

30. Environmental Protection Conservation and Protection, *Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Daphnia magna*, Ottawa:Environment Canada, 1990.

31. Fedorak, P.M. Microbial metabolism of organosulfur compounds in petroleum. In: *Geochemistry of Sulfur in Fossil Fuels*, edited by Orr, W.L. and White, C.M. Washington: American Chemical Society, 1989, p. 93-112.

32. Fedorak, P.M. and Grbic´-Galic´, D. Aerobic microbial cometablism of benzothiophene and 3-methylbenzothiophene. *Appl. Environ. Microbiol.* 57:932-940, 1991.

33. Fedorak, P.M., Payzant, J.D., Montgomery, D.S. and Westlake, D.W.S. Microbial degradation of *n*- alkyltetrahydrothiophenes found in petroleum. *Appl. Environ. Microbiol.* 54:1243-1248, 1988.

34. Fedorak, P.M. and Peakman, T.M. Aerobic microbial metabolism of some alkylthiophenes found in petroleum. *Biodegradation.* 2:223-236, 1992.

35. Fedorak, P.M. and Westlake, D.W.S. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. *Can. J. Microbiol.* 29:291-296, 1983.

36. Fedorak, P.M. and Westlake, D.W.S. Degradation of sulfur heterocycles in Prudhoe Bay crude oil by soil enrichments. *Water Air Soil Pollut.* 21:225-230, 1983.

37. Firth, B.K. and Backman, C.J. Comparison of Microtox testing with rainbow trout (Acute) and *Ceriodaphnia* (Chronic) bioassays in mill wastewaters. *Tappi Journal* December:169-174, 1990.

38. Foght, J.M., Fedorak, P.M., Gray, M.R. and Westlake, D.W.S. Microbial desulfurization of petroleum. In: *Microbial Mineral Recovery*, edited by Ehrlich, H.L. and Brierley, C.L. New York: McGraw-Hill Publishing Company, 1990, p. 379-407.

39. Foght, J.M. and Westlake, D.W.S. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. *Can. J. Microbiol.* 34:1135-1141, 1988.

40. Friocourt, M.P., Berthou, F. and Picart, D. Dibenzothiophene derivatives as organic markers of oil pollution. *Toxicol. Environ. Chem.* 5:205-215, 1982.

41. Grahl-Nielsen, O., Staveland, J.T. and Wilhelmsen, S. Aromatic hydrocarbons in benthic organisms from coastal areas polluted by Iranian crude oil. *J. Fish. Res. Board Can.* 35:615-623, 1978.

42. Gälli, R., Rich, H.W. and Scholtz, R. Toxicity of organophosphate insecticides and their metabolites to the water flea *Daphnia magna*, the Microtox test and an acetylcholinesterase inhibition test. *Aquat. Toxicol.* 30:259-269, 1994.

43. Hankenson, K. and Schaeffer, D.J. Microtox assay of trinitrotoluene, diaminonitrotoluene,and dinitromethylaniline mixtures. *Bull. Environ. Contam. Toxicol.* 46:550-553, 1991.

44. Hannoun, M., Blazevic, N., Kolbah, D., Mihalic, M. and Kajfez, F. *alpha* -Phenylpropionic acid derivatives. Synthesis and dethiation of 5-benzo[*b*]thiophene-3-carboxylic acid. *J. Heterocyclic Chem.* 19:1131-1136, 198.

45. Ho, K.T.Y. and Quinn, J.G. Physical and chemical parameters of sediment extraction and fractionation that influence toxicity, as evaluated by Microtox. *Environ. Toxicol. Chem.* 12:615-625, 1993.

46. Hoke, R.A., Giesy, J.P. and Kreis Jr., R.G. Sediment pore water toxicity identification in the lower Fox River and Green Bay, Wisconsin, using the Microtox assay. *Ecotox. Environ. Saf.* 23:343-354, 1992.

47. Horowitz, A. and Atlas, R.M. Continuous open flow-through system as a model for oil degradation in the Arctic Ocean. *Appl. Environ. Microbiol.* 33:647-653, 1977.

48. Hunt, J.M. *Petroleum Geochemistry and Geology*. San Francisco:. W.H. Freeman and Co., 1979.

49. Jacob, J. *Sulfur Analogues of Polycyclic Aromatic Hydrocarbons (Thiaarenes)*, Cambridge:Cambridge University Press, 1990.

50. Jacob, J., Schmoldt, A. and Grimmer, G. The predominant role of S-oxidation in rat liver metabolism of thiaarenes. *Canc. Lett.* 32:107-116, 1986.

51. Jacobs, M.W., Coates, J.A., Delfino, J.J., Bitton, G., Davis, W.M. and Garcia, K.L. Comparison of sediment extract Microtox toxicity with semi-volatile organic priority pollutant concentrations. *Arch. Environ. Contam. Toxicol.* 24:461-468, 1993.

52. Jacobs, M.W., Delfino, J.J. and Bitton, G. The toxicity of sulfur to Microtox from acetonitrile extracts of contaminated sediments. *Environ. Toxicol. Chem.* 11:1137-1143, 1992.

53. Jamison, V.M., Raymond, R.L. and Hudson, J.O. Biodegradation of high-octane gasoline in groundwater. *Dev. Ind. Microbiol.* 16:305-312, 1975.

54. Jobson, A., McLaughlin, M., Cook, F.D. and Westlake, D.W.S. Effect of amendments on the microbial utilization of oil applied to soil. *Appl. Microbiol.* 27:166-171, 1974.

55. Kaiser, K.L.E. and Palabrica, V.S. *Photobacterium phosphoreum* toxicity data index. *Water Pollut. Res. J. Can.* 26:361-431, 1991.

56. Karcher, W., Nelen, A., Depaus, R., van Eijk, J., Glaude, P. and Jacob, J. New results in the detection, identification and mutagenic testing of heterocyclic polycyclic aromatic hydrocarbons. In: *Chemical Analysis and Biological Fate: Polynuclear Aromatic Hydrocarbons*, edited by Cooke, M. and Dennis, A.D. Columbus: Battelle Press, 1981, p. 317-327.

57. Kinney, P.J., Button, D.K. and Schell, D.M. Kinetics of dissipation and biodegradation of crude oil in Alaska's Cook Inlet. In: *Proceedings of 1969 Joint Conference on Prevention and Control of Oil Spills*. Washington: American Petroleum Institute, 1969, p. 333-340.

58. Kodama, K., Nakatani, S., Umehara, K., Shimizu, K., Minoda, Y. and Yamada, K. Microbial conversion of petro-sulfur compounds: Part III. Isolation and identification of products from dibenzothiophene. *Agr. Biol. Chem.* 34:1320-1324, 1970.

59. Kodama, K., Umehara, K., Shimizu, K., Nakatani, S., Minoda, Y. and Yamada, K. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. *Agr. Biol. Chem.* 37:45-50, 1973.

60. Kropp, K.G., Gonçalves, J.A., Anderson, J.T. and Fedorak, P.M. Bacterial transformations of benzothiophene and methylbenzothiophenes. *Environ. Sci. Technol.* 28:1348-1356, 1994.

61. Kropp, K.G., Gonçalves, J.A., Andersson, J.T. and Fedorak, P.M. Microbially mediated formation of benzonaphthothiophenes from benzo[b]thiophenes. *Appl. Environ. Microbiol.* 60:3624-3631, 1994.

62. Laborde, A.L. and Gibson, D.T. Metabolism of dibenzothiophene by a *Beijerinckia* species. *Appl. Environ. Microbiol.* 34:783-790, 1977.

63. Lagno, Z.Y. and Sviridov, S.M. Toxicity of benzothiophene and benzothiophene sulfone. *Khim. Seraorg. Soedin. Soderzh. Neftyakh. Nefteprod.* 9:580-583, 1972. *Chem. Abs.* 82:119738e, 1975.

64. Leahy, J.G. and Colwell, R.R. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* 54:305-315, 1990.

65. MacKay, D. and Shiu, W.Y. Aqueous solubility of polynuclear aromatic hydrocarbons. *J. Chem. Eng. Data* 22:399-402, 1977.

66 MacKay, D. and Shiu, W.Y. *Illustrated Handbook of Physical-Chemical Properties and Environmental Fate of Organic Chemicals.* Chelsea. Lewis Publishers, 1993.

67. Mazidji, C.N., Koopman, B., Bitton, G., Voiland, G. and Logue, C. Use of Microtox and *Ceriodaphnia* bioassays in wastewater fractionation. *Toxic. Asses.* 5:265-277, 1990.

68. McFall, T., Booth, G.M., Lee, M.L. Mutagenic activity of methyl-substituted tri- and tetracyclic aromatic sulfur heterocycles. *Mut. Res.* 135:97-103, 1984.

69. Microbics Corporation, *Microtox Manual*, Carlsbad, CA:Microbics Corporation, 1992.

70. Middaugh, D.P., Resnick, S.M., Lantz, S.E, Heard, C.S. and Mueller, J.G. Toxicological assessment of biodegraded pentachlorophenol: Microtox and fish embryos. *Arch. Environ. Contam. Toxicol.* 24:165-172, 1993.

71. Monticello, D.J., Bakker, D. and Finnerty, W.R. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. *Appl. Environ. Microbiol.* 49:756-760, 1985.

72. Mueller, J.G., Middaugh, D.P., Lantz, S.E. and Chapman, P.J. Biodegradation of creosote and pentachlorophenol in contaminated groundwater: chemical and biological assessment. *Appl. Environ. Microbiol.* 57:1277-1285, 1991.

73. Munkittrick, K.R., Power, E.A. and Sergy, G.A. The relative sensitivity of Microtox, Daphnid, rainbow trout, and fathead minnow acute lethality tests. *Environ. Toxicol. Water Qual.* 6:35-62, 1991.

74. OECD Guidelines for Testing of Chemicals, *Water Solubility*, New York: Organization for Economic Co-operation and Development, 1981.

75. Ogata, M. and Fujisawa, K. Organic sulfur compounds and polycyclic hydrocarbons transferred to oyster and mussel from petroleum suspension. *Water Res.* 19:107-118, 1985.

76. Orr, W.L. *Oil Sand and Oil Shale Chemistry*, New York:Verlag Chemie, 1978.

77. Pelroy, R.A., Stewart, D.L., Tominaga, Y., Iwao, M., Castle, R.N. and Lee, M.L. Microbial mutagenicity of 3- and 4-ring polycyclic aromatic sulfur heterocycles. *Mut. Res.* 117:31-40, 1983.

78. Perry, J.J. Microbial metabolism of cyclic alkanes. In: *Petroleum Microbiology*, edited by Atlas, R.M. New York: Macmillan Publishing Co., 1984, p. 61-97.

79. Prince, R.C. Bioremediation of oil spills, with particular reference to the spill from the Exxon Valdez. In: *Microbial Control of Pollution*, edited by Fry, J.C., Gadd, G.M., Herbert, R.A., Jones, C.W. and Watson-Craik, I.A. Bath: Cambridge University Press, 1992, p. 19-34.

80. Rall, H.T., Thompson, C.J., Coleman, H.J. and Hopkins, R.L. *Sulfur Compounds in Crude oil*, Washington: U.S. Dept. of Interior, Bureau of Mines, 1972.

81. Rand, G.M and Petrocelli, S.R. Introduction. In: *Fundamentals of Aquatic Toxicology*, edited by Rand, G.M. and Petrocelli, S.R. Washington: Hemisphere Publishing Corporation, 1985, p. 1-30.

82. Saftic´, S., Fedorak, P.M. and Andersson, J.T. Diones, sulfoxides, and sulfones from the aerobic cometabolism of methylbenzothiophenes by *Pseudomonas* strain BT1. *Environ. Sci. Technol.* 26:1759-1764, 1992.

83. Saftic´, S., Fedorak, P.M. and Andersson, J.T. Transformation of methylbenzothiophenes by three *Pseudomonas* isolates. *Environ. Sci. Technol.* 27:2577-2584, 1993.

84. Schlesinger, A.H. and Mowry, D.T. Benzothiophenes and their 1-dioxides. *J. Amer. Chem. Soc.* 75:2614-2615, 1957.

85. Sergy, G. and Scroggins, R. *Biological Test Method: Acute Lethality Test Using Daphnia spp*, Ottawa: Environmment Canada, 1990.

86. Singer, M.E. and Finnerty, W.R. Microbial metabolism of straight-chain and branched alkanes. In: *Petroleum Microbiology*, edited by Atlas, R.M. New York: MacMillian Publishing Co., 1984, p. 1-59.

87. Somasundaram, L., Coats, J.R., Racke, K.D. and Stahr, H.M. Application of the Microtox system to assess the toxicity of pesticides and their hydrolysis metabolites. *Bull. Environ. Contam. Toxicol.* 44:254-259, 1990.

88. Spain, J.C., Pritchard, P.H. and Bourquin, A.W. Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. *Appl. Environ. Microbiol.* 40:726-734, 1980.

89. Spain, J.C. and Van Veld, P.A. Adaptation of natural microbial communities to degradation of xenobiotic compounds: Effects of concentration, exposure time, inoculum, and chemical structure. *Appl. Environ. Microbiol.* 45:428-435, 1983.

90. Speight, J.G. *The Chemistry and Technology of Petroleum*, New York:Marcel Dekker, Inc., 1980.

91. Symons, B.D. and Sims, R.C. Assessing detoxification of a complex hazardous waste, using the microtox bioassay. *Arch. Environ. Contam. Toxicol.* 17:497-505, 1988.

92. Takata, T., Yamazak, M., Fujimari, K., Kim, Y.H., Iyanagi, T. and Oae, S. Enzymatic oxidation of sulfides with cytochrome P-450 from rabbit liver. *Bull. Chem. Soc. Jpn.* 56:2300-2310, 1983.

93. Talak, B.D. Carcinogenesis by thiophene isomers of polycyclic hydrocarbons. *Tetrahedron.* 9:76-95, 1960.

94. Vassilaros, D.L., Eastmond, D.A., West, W.R., Booth, G.M. and Lee, M.L. Determination and bioconcentration of polycyclic aromatic sulfur in heterocycles in aquatic biota. In: *Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry*, edited by Cooke, M., Dennis, A.J. and Fisher, G.L. Columbus: Battelle Press, 1982, p. 845-857.

95. Weast, R.C. *CRC Handbook of Chemistry and Physics* 49th Edition, Cleveland, OH: Chemical Rubber Company, 1968. Ed. 49

96. Zhou, E. and Crawford, R.L. Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. *Biodegradation.* 6:127-140, 1995.